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(54) Title: LKP PILIN STRUCTURAL GENES AND OPERON OF NONTYPABLE HAEMOPHILUS INFLUENZAE

(57) Abstract

The invention relates to the isolation and cloning of the structural gene, *hip*P, for the NTHi pili serotype 5 and the LKP operon. The invention relates to DNA molecules capable of hybridizing to the DNA sequences of the *Haemophilus influenzae* genome related to the pili. The invention further relates to a DNA molecule which encodes a pili protein, particularly a tip adhesin protein. The DNA molecules of the invention can be used in a method for assaying a sample, such as a blood sample, for the presence of *Haemophilus influenzae* in the sample. Accordingly, the invention further relates to the use of the DNA molecules as a diagnostic. The invention also relates to a recombinant *Haemophilus influenzae* pili protein, such as a tip adhesin protein. The protein can be employed in a method for immunizing an animal, such as a human, as a therapeutic or diagnostic.

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LKP PILIN STRUCTURAL GENES AND OPERON OF NONTYPABLE *HAEMOPHILUS INFLUENZAE*

Background of the Invention

Nontypable *Haemophilus influenzae* (NTHi) are primarily noninvasive human respiratory tract pathogens. NTHi can reside in the respiratory tract as a commensal or give rise to local infections, including otitis media, bronchitis, sinusitis, and rarely, pneumonia (Bluestone, C.D., and J.O. Klein, *In Pediatric Otolaryngology., 356* (1983); Bluestone and Stool ed. W.B. Saunders Co. Philadelphia.; Musher, D.M. *et al., Ann. Intern. Med. 99*:344-350 (1983)). Several potential adherence factors have been described for *Haemophilus influenzae* (both typable and nontypable) adherence to human cells, including four classes of fimbriae/pili and two high molecular weight proteins with similarity to the filamentous hemagglutinin of *Bordetella pertussis* (St. Geme, J.W., *et al., Proc. Natl. Acad. Sci. USA 90*:2875-2879 (1993)). Pili are bacterial surface antigens. They are protein appendages consisting of a helically symmetrical assembly of major protein (pilin) subunits. Some pili can also carry from two to three minor proteins assembled on their tips. One of these proteins, adhesin, carries the active site for pilus adhesion to specific membrane receptors on human and animal cells.

One class of pili/fimbriae has been widely studied, the <u>l</u>ong thic<u>k</u> <u>p</u>ili (LKP) family. LKP pili are expressed by both typable and nontypable *H. influenzae* (Hib). The pili in this family have a characteristic morphology, partially shared adhesion specificity and their structural proteins share amino acid sequences. These pili are hemagglutination positive and mediate attachment to human mucosal cells (Brinton, C.C. *et al., Pediatr. Infect. Dis.*

mucosal cells (Brinton, C.C. *et al.*, *Pediatr. Infect. Dis. J. 8 Suppl.*:54-61 (1989)). Hemagglutination of human erythrocytes is accomplished via binding to the AnWj blood group antigen while binding to epithelial cells involves a sialic acid containing lactosylceramide receptor (van Alphen, L. *et al.*, *Infect. Immun. 69*:4473-4477 (1991)).

The LKP family has been divided into different strain specific serotypes based on reactivity to polyclonal antisera raised against the purified pili. Little cross reactivity among pili serotypes has been observed (Brinton, C.C., *et al.*, *Pediatr. Infect. Dis. J. 8 Suppl.*:54-61 (1989)).

Inhibiting, or blocking, LKP pilus-mediated adhesion by *H. influenzae* to cells can prevent *H. influenzae* diseases. Purified, intact LKP pili have been shown to be vaccine candidates for NTHi otitis media in the chinchilla model, conferring protection against challenge with NTHi strains bearing homologous pili serotype (Karasic, R. *et al.*, *Pediatr. Infect. Dis. J. 8 (Suppl.)*: S62-65 (1988)). However, because protection is pilus-specific, for broad protection, a vaccine would be required to be multivalent, including the most frequently occurring serotypes of pili in the natural population of pathogens. LKP pilin structural genes have been cloned and sequenced by several groups (Coleman, T. *et al.*, *Infect. Immun. 59*:1716-1722 (1991); Forney, L.J. *et al.*, *Infect. Immun. 59*:1991-1996 (1991); Kar, S., *et al.Infect. Immun. 58*:903-908 (1990); van Ham, S.M., *et al.*, *EMBO Jour. 8*:3535-3540 (1989)), but only the genes responsible for pili serotypes 1 and 4 have been identified.

Summary of the Invention

The invention relates to the isolation, cloning and sequencing of the pilin gene for the *Haemophilus influenzae* pili serotype 5 (Figure 1), to the sequencing

of the entire LKP1 operon, which is set forth in Table 5 and to the cloning of the LKP10, LKP11, and LKP12 pili. The present invention also relates to DNA molecules (also referred to herein as DNA sequences or nucleic acid sequences) which encode proteins which comprise the *H. influenzae* LKP, particularly a tip adhesin protein. The present invention also relates to DNA molecules capable of hybridizing to the DNA sequences of the *Haemophilus influenzae* genome related to the pili. The DNA molecules of the present invention can be used in a method for assaying a sample, such as a blood sample, for the presence of *Haemophilus influenzae*. Accordingly, the present invention relates to the use of the DNA molecules as a diagnostic.

The present invention further relates to recombinant *Haemophilus influenzae* pili proteins, and peptides, specifically a tip adhesin protein. The proteins, or peptides, of the present invention can be used to produce antibodies, both polyclonal and monoclonal, which are reactive with (i.e., bind to) the *H. influenzae* pili proteins, and can be used in diagnostic assays to detect the presence of *Haemophilus influenzae* antibodies, in for example, a blood sample. Such antibodies to also be used as vaccines in methods of passive immunization.

The proteins and peptides of the present invention can also be employed in methods for immunizing a mammal, such as a human, against *Haemophilus influenzae* infection and, thus, as a vaccine for the prevention of *Haemophilus influenzae* related diseases, for example, otitis media. In particular, based on the DNA and amino acid sequences presented herein, an adhesin protein, or peptide, vaccine can be constructed which can induce protecting antibodies to *H. influenzae* in mammals.

Brief Description of the Drawings

Figure 1 is a graphic illustration of the conserved regions of the pilin genes of *H. influenzae* serotypes 1, 4 and 5 (SEQ ID NOs:1-3, respectively).

Figures 2A, 2B, and 2C are schematics of the physical maps obtained by restriction enzyme digestion of vectors containing LKP inserts.

Figure 3 shows the amino acid sequence of LKP1 fusion protein. The underline indicates the partial amino acid sequence of the LKP tip adhesin protein that was fused to maltose-binding protein.

Figure 4 is a photograph of a gel showing the identification of LKP1 tip adhesin protein by antibodies reactive with the fusion protein of LKP1 tip adhesin-MBP in Western blotted membranes. Lanes 1 and 2: different preps of purified LKP1 pili with tip protein (47Kd). (A positive reaction was shown between tip protein and the antibody); lane 3: purified LKP10 pili with tip adhesin (47Kd). (The tip protein does not react with the antibody); lane 4: purified LKP11 pili with tip protein (47Kd). (The tip protein does not react with the antibody); lane 5: protein molecular weight markers.

Figure 5 is a photograph of a gel showing the binding activity of LKP1 tip adhesin to human red cell (HRC) ghosts. Lane 1: molecular weight markers; lane 2: purified LKP1 pili with tip protein; lane 3: the pili with HRC ghosts after centrifugation. Tip protein band (47Kd) disappeared due to the binding of tip adhesin pili to ghosts pellet; lane 4: HRC ghosts after centrifugation, used as control; lane 5: purified pili without tip protein (treated with 1% SDS) was incubated with fresh ghosts, showing the same protein band pattern as the pattern of lane 3; Lane 6: purified pili without tip protein. Prior to the gel loading, pili were treated with 1% SDS, exhaustively dialyzed in 25 m Tris buffer, pH 8.0,

crystallized by PEG plus NaCl and resolubilized in 25 mM Tris buffer, pH 8.0.

Figure 6 is a photograph of a gel showing the binding activity of purified LKP1 tip adhesin protein to human red cell ghosts. Lane 1: molecular weight markers; lane 2: purified tip adhesin protein with a molecular weight of 47Kd and the protein was removed by 0.1% SDS in 100 mM Glycine buffer, pH 2.0; lane 3: purified adhesin was incubated with fresh human red cell ghosts and pelleted by centrifugation prior to loading the supernatant on the gel. The tip adhesin band disappeared due to the binding to HRC ghosts; lane 4: purified adhesin was incubated with boiled HRC ghosts and pelleted by centrifugation prior to loading the supernatant on the gel. It showed adhesin band with 47Kd, which indicates that tip adhesin protein does not bind to the ghosts pellet; lane 5: supernatant of fresh ghosts after centrifugation. It was used as a control; lane 6: supernatant of boiled HRC ghosts after centrifugation, showing a different soluble protein pattern from that of fresh HRC ghosts, used as another control; lane 7: different prep of purified tip protein incubated with fresh HRC ghosts, which showed the binding between tip protein and fresh HRC ghosts pellet; lane 8: different prep of purified tip protein incubated with boiled HRC ghosts, indicating that the tip protein does not bind the denatured ghosts. The gel was silver stained.

Figure 7 is a photograph of a gel showing adhesin proteins from different LKP type pili with the same molecular weight. Lane 1: molecular weight markers; lane 2: LKP10 pili; lane 3: LKP11 pili and lane 4 to 6: different purified preparation of LKP1 pili. Proteins were stained with silver.

Detailed Description of the Invention

Described herein, for the first time, is the cloning of the *Haemophilus influenzae* serotype 5 pilin gene and the sequence of the entire LKP1 operon (SEQ ID NO: 4) and the deduced amino acid sequences for six open reading frames (SEQ ID NOs: 5-10). The LKP1 operon, as shown in Table 5, is composed of five separate genes, designated *hipP* (the pilin gene), *hipC* (the periplasmic chaperone gene), *hipR* (the membrane anchor gene), *hipM* (the minor tip associated protein gene) and *hipA* (the tip adhesin gene). These five genes are also referred to herein as *hifA* (for *hipP*), *hifB* (for *hipC*), *hifC* (for *hipR*), *hifD* (for *hipM*) and *hifE* (for *hipA*). Also present on the LKP1 operon are an integrase gene, and a peptidase gene. The proteins encoded by these genes of the LKP1 operon and the LKP5 pilin protein are collectively referred to herein as the *H. influenzae* pili proteins.

The present invention encompasses the isolated and/or recombinant nucleic acid sequences encoding the *H. influenzae* pili proteins, or biologically active fragments thereof, described herein. As used herein nucleic acids are also referred to as DNA and RNA, or DNA sequences and RNA sequences, or DNA molecules or RNA molecules. Nucleic acids referred to herein as "isolated" are nucleic acids separated away from the nucleic acids of the genomic DNA or cellular RNA of their source of origin (e.g., as it exists in cells or in a mixture of nucleic acids such as a library), and may have undergone further processing. "Isolated" nucleic acids include nucleic acids obtained by methods known to those of skill in the art to obtain isolated nucleic acids and methods described herein. These isolated nucleic acids include essentially pure nucleic acids, nucleic acids produced by chemical synthesis, by combinations of biological and chemical methods, and recombinant nucleic acids which are isolated.

Nucleic acids referred to herein as "recombinant" are nucleic acids which have been produced by recombinant DNA methodology, including those nucleic acids that are generated by procedures which rely upon a method of artificial recombination, such as the polymerase chain reaction (PCR) and/or cloning into a vector using restriction enzymes. "Recombinant" nucleic acids are also those that result from recombination events that occur through the natural mechanisms of cells, but are selected for after the introduction to the cells of nucleic acids designed to allow and make probable a desired recombination event.

Also encompassed by the present invention are nucleic acid sequences (DNA or RNA sequences) which are substantially complementary to the *H. influenzae* DNA sequences described herein, and nucleic acid sequences which hybridize with these DNA sequences under conditions of stringency known to those of skill in the art sufficient to identify DNA sequences with substantial nucleic acid sequence identity. It is reasonable to predict that DNA sequences identified under such stringent conditions will likely encode a protein (also referenced to herein as a polypeptide, or peptide fragment) with the biological activity of *H. influenzae* pili proteins. A general description of stringent hybridization conditions are discussed in Ausubel, F.M., *et al.*, Current Protocols in Molecular Biology, Greene Publishing Assoc. and Wiley-Interscience 1989, the teachings of which are incorporated herein by reference. Factors such as probe length, base composition, percent mismatch between the hybridizing sequences, temperature and ionic strength influence the stability of nucleic acid hybrids. Thus, stringency conditions sufficient to identify additional *H. influenzae* pili proteins, (e.g., high or moderate stringency conditions) can be determined empirically, depending in

part upon the characteristics of the known DNA to which other unknown nucleic acids are being compared for sequence similarity.

As defined herein, substantially complementary means that the sequence need not reflect the exact sequence of e.g., SEQ ID NO:4, but must be sufficiently similar in identity of sequence to hybridize with SEQ ID NO:4 under stringent conditions. For example, non-complementary bases, or longer or shorter sequences can be interspersed in sequences provided the sequence has sufficient complementary bases with, e.g., SEQ ID NO:4 to hybridize therewith.

The DNA molecules of the present invention can, preferably, encode a functional or biologically active pili protein, such as the pilin gene, *hipP*; the periplasmic chaperon, *hipC*; the membrane anchor protein, *hipR*; the tip associated protein, *hipM* and most preferably, the tip adhesin protein, *hipA*. A "functional or biologically active protein" is defined herein as a protein which shares signicant identity (e.g., at least about 65%, preferably at least about 80% and most preferably at least about 95%) with the corresponding sequences of the endogenous protein and possesses one or more of the functions thereof. Biological functions of the *H. influenzae* pili proteins include antigenic structural, and adhesion properties. For example, as described in Karasic, R. *et al.* (Karasic, R. *et al.*, *Pediatr. Infect. Dis. J. 8 (Suppl.)*: S62-65 (1988)), the teachings of which are herein incorporated by reference, pili proteins can be shown to adhere to mucosal cells and erythrocytes. Thus, such adhesion properties can be a measure of biological activity. Also described herein, biological activity can include the antigenicity of the protein, or peptide, resulting in the production of antibodies which bind to the pili proteins.

The *H. influenzae* pili proteins of the present invention are understood to specically include the proteins of the LKP1 operon and the serotype 5 *hipP* pilin protein, and proteins having amino acid sequences analogous to these sequences. Such proteins are defined herein as *H. influenzae* pili protein analogs, or derivatives. Analogous amino acid sequences are defined herein to mean amino acid sequences with sufficient identity of amino acid sequence with, e.g., LKP1 tip adhesin protein, to possess the biological activity of tip adhesin. The biological activity of tip adhesin can include, for example, the capability of tip adhesin to bind to specic membrane receptors on human and animal cells. For example, an analog polypeptide can be produced with "silent" changes in the amino acid sequence wherein one, or more amino acid residue differs from the amino acid residues of the LKP1 adhesin, yet still possess adhesion activity. Examples of such differences include additions, deletions or substitutions of residues to e.g., SEQ ID NO:9. Also encompassed by the present invention are analogous proteins that exhibit lesser or greater biological activity of the pili proteins of the present invention.

The present invention also encompasses biologically active protein, or biologically active fragments of the *H. influenzae* pili proteins described herein. Such fragments can include only a part of the full-length amino acid sequence of a pili protein yet possess biological activity. Such fragments can be produced by amino- and carboxyl-terminal deletions, as well as internal deletions. Such peptide fragments can be tested for biological activity as described herein. Thus, a functional, or biologically active, protein includes mutants or derivatives of the endogenous protein wherein one or more amino acids have been substituted, deleted or

added. Also included are active fragments of the protein. The *H. influenzae* pili proteins, as set forth above, include functional or biologically active pili proteins, such as the pilin structural protein, *hipP;* the periplasmic chaperon, *hipC;* the membrane anchor protein, *hipR;* the tip associated protein, *hipM;* and most preferably, the tip adhesion protein, *hipA*.

The present invention further relates to fusion proteins comprising the pili proteins described herein (referred to herein as a first moiety) linked to a second moiety not occurring in the pili protein as found in nature. Thus, the second moiety can be a single amino acid, peptide or polypeptide. The first moiety can be in an N-terminal location, a C-terminal location or internal to the fusion protein. In one embodiment, the fusion protein comprises a pili protein and either a maltose binding protein (MBP) (SEQ ID NO:11) or glutathione-S-transferase (GST).

The DNA sequences of the present invention can also be used in a recombinant construct for the infection, transfection or transformation of a cell *in vitro* or *in vivo* under control of an appropriate promoter for the expression of functional *H. influenzae pili* proteins, as defined herein, in an appropriate host cell. Such recombinant constructs are also referred to herein as expression vectors. For example, a DNA sequence can be functionally ligated to a suitable promoter (e.g., a constitutive or inducible promoter or the endogenous promoter) introduced into a suitable expression vector, such as pUC19, which is then introduced into a suitable host cell. The construct can also include DNA encoding one or more selectable markers (such as neo, gpt, dhfr, ada, pac, hyg and hisd) or DNA encoding one or more different antigens or therapeutic proteins.

The construct can be introduced by any suitable means, as set forth above, such as by calcium phosphate precipitation, microinjection, electroporation or infection (such as with an infectious retroviral, herpes vaccinia or adenovirus vector). The host cell can be a eucaryotic or procaryotic cell. Suitable cells include bacterial (e.g. *E. coli*) or mammalian cells. Mammalian cells include primary somatic cells, such as, epithelial cells, fibroblasts, keratinocytes, macrophages or T cells, or immortalized cell lines, such as HeLa or HT1080. The recombinant host cell can then be cultured and, optionally, selected, *in vitro* under appropriate conditions resulting in the expression of the protein. Alternatively, the cell can be transplanted or injected into an animal, such as a human, for *in vivo* expression.

In one embodiment, the present invention relates to LKP type pili-producing *E. coli* recombinants. Such recombinants have been constructed from *Haemophilus infuenzae*, as described herein. These single serotype recombinants produced pili in large, easily puriable quantities. They did not phase vary or become recalcitrant upon subculture and could be grown as *E. coli* in liquid medium with good pilus yields. The single serotype pilus preparations grown and puried from them contained pili identical to those on the parent *H. influenzae* (*Hflu*) strains and contained no other *Hflu* antigens. These preparations are easily standardized for purity, identity, concentration and potency for subsequent mixing into a multivalent vaccine and provides an efficient means of producing pilus for vaccine manufacture. As described herein, single-type-producing *E. coli* recombinant vaccine strains have been constructed for LKP10, LKP11 and LKP12 serotypes.

Multiple serotype recombinants containing two operons on separate plasmids have also been constructed. Single

colonies of these strains simultaneously expressed, in good quantities, two serotypes of pili. However, these strains were unstable in that, during *in vitro* subculture, they tended to rapidly lose pilus expression, perhaps because the plasmids used were incompatible. When the two operons are placed on two compatible plasmids these strains are expected to be more stable. The use of stable, high-producing double-expressing recombinant strains could simply production of proteins suitable for vaccine use by reducing by half the number of vaccine strains required.

Good production, concentration and purication methods for *Hflu* LKP pili of different serotypes have been developed and are described herein. Pili can be puried from *E. coli* recombinant cultures producing *Hflu* pili as described for the purication of pili from *Hflu* culture. Both solid phase and liquid phase fermentation methods have been used. The preferred procedure involves mechanical removal of pili from the harvested bacteria and their separation from the bacterial cells by centrugation. Pili are concentrated and further puried by alternate cycles of longitudinal aggregation (crystallization) of intact pilus rods with soluble impurities removed by centrugation of the crystals followed by solubilization of the pilus crystals into free pilus rods with particulate impurities removed by centrugation. Each stage of the production/purication process was optimized for each pilus serotype. To date, nineteen different LKP serotypes have been puried.

Alternative pilus purication methods with analytical and industrial utility have also been developed Using appropriate solvent and column conditions, intact pili can be puried away from contaminating proteins by HPLC or FPLC on molecular sizing, hydrophobic or ion exchange columns.

These methods are also capable of scale-up for industrial production.

Purication methods for individual pilus proteins have also been developed starting with intact LKP pili. *Hflu* LKP pilus structural proteins, as deduced from the multiple sequence alignment of pilus gene sequences with other pilus genes, include pilin, small tip minor and large tip minor proteins. The large tip minor protein is referred to as the "adhesin" because it carries the known LKP pilus adhesion specicity for human red blood cells. However, by analogy with other pilus families, the other two LKP pilus structural proteins may also be adhesins with specicities for as yet unknown human receptors. Both pilins and adhesins of LKP pili have been puried in biologically active form.

The pilins are puried in assembled rod form by removal of the minor tip proteins and separation of rods from minors on molecular sizing columns. In their assembled form, the pilin units retain the antigenic specicity of intact pili which is conferred by the exposed surface determinants of the pilin subunits on the lateral surface of the pilus rod. Pilin rods are expected to be equally as effective multivalent vaccine components as intact pili may have advantage of higher purity and possibly reduced side effects.

The adhesin of LKP11 has been isolated and puried in active and soluble form. Its removal from LKP11 pili eliminates the ability of these pili to bind to human red blood cells. In pure form it can bind to human red blood cell membranes. The adhesin band on SDS gels is labeled by antibodies reactive with fusion protein comprised of a fragment of adhesin and maltose binding protein. Purified LKP pilus adhesins may have utility as vaccine components capable of inducing adhesion-blocking or clearing antibodies. The LKP11 adhesin did not cross-react

antigenically with the LKP1 adhesin on Western blots. Thus, the SDS/PAGE gel similarity of apparent molecular weights found for 3 different LKP adhesins was not predictive of antigenic similarity in this limited two-serotype test. Free adhesins can be tested for efficacy as otitis media vaccines and for their ability to induce adhesion-blocking antibodies. Antiserum to the fusion protein, which labeled the adhesin band on Western blots, did not block adhesion to red cells.

The isolated recombinant proteins of the present invention can be administered to a mammal to protect, or to treat the mammal against *H. influenzae* infection. Isolated recombinant pili protein can be formulated into a vaccine composition, for example, as described in U.S. Patent 5,336,490, the teachings of which are incorporated herein by reference. The protein can also be administered via an infectious construct, preferably a replication incompetent or attenuated viral construct. Alternatively, the protein can be administered via a recombinant host cell (such as, a mammalian cell) which will express the protein *in vivo* or in a pharmaceutically acceptable carrier. In particular, the recombinant LKP1 tip adhesin protein, a biologically active fragment thereof, or a fusion protein, can be used in a vaccine composition to induce the production of antibodies in a mammal. It is reasonable to predict that such antibodies can protect the mammal from *H. influenzae* diseases.

The vaccine composition may be administered in a singel dose or in more than one dose over a period of time to achieve a level of antibody in the blood which is sufficient to confer protection from *H. influenzae* infection.

Suitable pharmaceutical carriers include, but are not limited to water, salt solutions, alcohols, polyethylene glycols, gelatin, carbohydrates such as lactose, amylose

or starch, magnesium stearate, talc, silicic acid, viscous paraffin, fatty acid esters, hydroxymethylcellulose, polyvinyl pyrolidone, etc. The pharmaceutical preparations can be sterilized and desired, mixed with auxiliary agents, e.g., lubricants, preservatives, stabilizers, wetting agents, emulsiers, salts for influencing osmotic pressure, buffers, coloring, and/or aromatic substances and the like which do not deleteriously react with the active compounds. They can also be combined where desired with other active agents, e.g., enzyme inhibitors, to reduce metabolic degradation.

For parenteral application, particularly suitable are injectable, sterile solutions, preferably oily or aqueous solutions, as well as suspensions, emulsions, or implants, including suppositories. Ampoules are convenient unit dosages.

Modes of administration are those known in the art, such as parenteral, oral or intranasal administration or by cellular implantation.

It will be appreciated that the actual effective amounts of the protein in a specic case will vary according to the specic compound being utilized, the particular composition formulated, the mode of administration and the age, weight and condition of the patient, for example. As used herein, an effective amount of protein is an amount of protein which is capable of raising the level of antibody in a mammal to a level sufficient to provide protection from *H. influenzae* infection. Dosages for a particular patient can be determined by one of ordinary skill in the art using conventional considerations, (e.g. by means of an appropriate, conventional pharmacological protocol).

The DNA molecules and proteins of the present invention can be used in *in vitro* diagnostic assays to detect the presence of *H. influenzae* in biological

samples. In one embodiment, the DNA molecules, or fragments thereof, can be used as probes in an assay for detecting *Haemophilius influenzae* in a sample, such as a blood sample from a mammal, e.g. a human. Such probes can be designed such that they specically bind to the target sequence (e.g., an *H. influenzae* pili protein).

In one embodiment the DNA probe can comprise the nucleotides of a serotype conserved region of the *H. influenzae* genome, such as the nucleotides encoding a tip adhesin protein. To specically bind to the target sequence, the probe must be of sufficient length to provide the desired specicity, i.e., to avoid being hybridized to random sequences in the sample. The DNA molecule capable of hybridization preferably contains at least about 400 nucleotides, more preferably at least about 1000 nucleotides, and most preferably at least about 1200 nucleotides. For example, the DNA molecule can comprise at least about 400 nucleotides between about nucleotide 7000 to 7400 of SEQ ID NO:4. The DNA hybridization probe preferably shares at least about around 70% homology or the corresponding sequences of the *Haemophilus influenzae* genome, more preferably at least about 80% and most preferably at least about 90%.

In particular, the DNA molecules of the present invention are capable of hybridizing to serotype conserved regions of the *H. influenzae* genome. A particularly preferred embodiment are DNA molecules that hybridize with the *H. influenzae* region encoding the tip adhesin protein. For example, a DNA molecule can be capable of hybridizing to the gene encoding the tip adhesin protein of serotype 1, preferably the sequence set forth between about nucleotide 6955 to 8265 of SEQ ID NO:4. In one embodiment, the DNA molecule is capable of hybridizing to the genome under stringent conditions, as described herein. The hybridization assay can be performed

employing known hybridization procedures, such as those described herein. The probe can be, for example, detectably labeled employing known labels in the art, including enzymes, dyes, antibodies and radioactive labels. The probe is preferably immobilized on a solid support (e.g., a membrane).

Alternatively, the DNA molecule can be selected such that it hybridizes to a non-conserved region of the *Haemophilus influenzae* genome. For example, a DNA molecule that hybridizes to the gene encoding the pilin protein can be employed. Such an assay can detect the presence of a particular serotype of *Haemophilus influenzae* in the sample.

A sample which can be subjected to the present assay can be any sample which is suspected of containing or being contaminated with *Haemophilus influenzae*. Examples of such an sample include a blood sample, a nasopharyngeal sample, or an ear aspirate.

The assay can be used, therefore, as a diagnostic for the detection of infection of a subject, such as a mammal (e.g., a human), with *Haemophilus influenzae*. The assay can also be used to detect the presence of contamination of a material with *Haemophilus influenzae*, such as a food, medicament, or biological material.

In another embodiment, the protein can be used in an assay for detecting *Haemophilus influenzae* infection in a sample, such as a blood sample. For example, the pili of a pathogen can be isolated from the sample or recombinantly produced, employing the techniques described herein. One or more of the proteins, or fragments thereof, of the pili can then be sequenced. The sequences can be aligned to and compared with the corresponding protein sequence(s) of SEQ ID NO:4. Homology in excess of 90%, for example, is indicative of presence of the pathogen (i.e., infection) in the sample.

The pili protein, or a fragment thereof (e.g., a peptide fragment) can also be used in an immunoassay, specically an ELISA, to detect the presence of antibodies in biological samples (e.g., blood, serum or tissue). Such immunoassay can be readily performed by those of skill in the art using well-established techniques to detect antibody bound to LKP pili protein or peptide fragments.

The pili proteins, or fragments thereof (also referred to herein as peptides, or peptide fragments), can also be used to produce antibodies that are reactive with the pili proteins described herein. The term antibody is intended to encompass both polyclonal and monoclonal antibodies. Polyclonal antibodies can be prepared by immunizing an animal with a preparation of crude or puried pili protein using techniques well-known to those of skill in the art. Pili fusion proteins can also be used for immunization. Monoclonal antibodies can be prepared using techniques known to those of skill in the art. These antibodies can be used in diagnostic assays to detect the presence of *H. influenzae* antibodies in biological samples as described above.

The invention is further specically illustrated by the following examples.

Example 1: Cloning and Sequencing of the LKP 5 hipP gene and the LKP1 Operon

Materials and Methods

Bacterial strains and plasmids

H. influenzae strains P860295, P861249, and P860688 which express LKP serotypes 1, 4, and 5 respectively, described previously (Brinton, C.C. *et al., Pediatr. Infect. Dis. J. 8 Suppl.*: 54-61 (1989)) were employed. *E. coli* strains MB392 (Kar, S. *et al., Infect. Immun. 58*:903-

908 (1990)) and HB101 were used as hosts for recombinant plasmids and strain DH5-α was used for cloning steps involving β-galactosidase α-peptide complementation. *Hflu* were grown in brain heart infusion (Dco Laboratories, Detroit, MI) containing 10 μg/ml hemin (Sigma Chemical Co., St. Louis, MO) and 2 μg/ml NAD (Sigma) at 37° C. *E. coli* strains were grown in Luria broth (Miller, J.H., In *Experiments in molecular genetics., 203* (1972). Cold Spring Harbor Laboratory. Cold Spring Harbor, NY) at 37°C. Where appropriate, antibiotics were used at the following concentrations: ampicillin (Sigma) 100 μg/ml, kanamycin (Sigma) 25 μg/ml, and chloramphenicol (Sigma) 20 μg/ml.

Construction and properties of plasmid pHF1 which expresses LKP1 pili in *E. coli* as described previously (Kar, S. *et al.*, *Infect. Immun. 58*:903-908 (1990)) were employed. Plasmid pPX551 is a pUC18 derivative containing the 1.9 kb XhoI fragment of pHF1 inserted into the BamHI site. Deletion clones of pHF1 lacking the pepN locus were constructed as described in the text. The LKP4 pilin structural gene was isolated by PCR amplication of P860295 chromosomal DNA using primers with the following sequences: for the 5' end of the gene- 5'GTGCTGGATCCGTTTCTCTTGCATTACATTAGG 3' (SEQ ID NO:12) and for the 3' end- 5'TTAGGAATTCGGAAGCGTTTTTACTTTTTTTGG3' (SEQ ID NO:13). The 5' primer included a *Hind*III restriction site, underlined in the sequence, and the 3' primer included an *Eco*RI site also shown underlined. The PCR product was cloned into pCR1000 (Invitrogen, Inc., CA) as per manufacturer's directions. The LKP4 structural gene was subcloned by blunting the *Eco*RI site with Klenow in the presence of all four dNTPs, and cutting with *Asp*718 I (an *Asp*718 I site is located in the vector) releasing the fragment. The LKP4 gene was ligated into *Hind*II-*Asp*718 I cut pPX191 (a derivative of pUC19 with the *bla* gene replaced by the *cat* gene from pACYC184 (Chang,

A.C.Y., and S.N. Cohen, *J. Bacteriol. 134*:1141-1156 (1978)) to form pPX602.

The LKP5 pilin structural gene was isolated by PCR using the following primers: for the 5'end- 5'-AACGAATTCTGCTGTTTATTAAGGCTTTAG (SEQ ID NO:14) and for the 3'-AGCTGGATCCTTGTAGGGTGGGCGTAAGCC (SEQ ID NO:15). The PCR product of approximately 1 kb was cloned into pCRII (Invitrogen, Inc., San Diego, CA and subcloned as a blunt ended fragment by Klenow treatment of *Eco*RI ends generated using the vector's flanking *Eco*RI sites. The LKP5 pilin gene was subcloned into plasmid pPX191 and orientation determined by restriction analysis. The LKP5 subclone was saved as pPX605.

Cloning of *hipP* genes encoding other LKP serotypes

hipP loci encoding serotype 4 and serotype 1 LKP genes have been described (Kar, S. *et al.*, *Infect. Immun. 58*:903-908 (1990); van Ham, S.M. *et al.*, *EMBO Jour. 8*:3535-3540 (1989)). To determine the serotype specicity of LKP pili is located within the *hipP* gene, PCR was used to clone the serotypes 4 and 5 pilin genes from an NTHi strains expressing these pili. The PCR product for the LKP4 pilin gene was cloned into pPX191 as described above and is expressed under control of the lac promoter. The *hipP* gene from an LKP5 expressing *Hflu* strain was isolated by PCR as described and cloned into pPX191 for expression under lac control.

Oligonucleotide synthesis

The synthetic oligonucleotides used as primers for PCR amplication and DNA sequencing were synthesized on an Applied Biosystems (ABI) 380B DNA synthesizer using b-cyanoethyl phosphoramidite chemistry (Sinha, N.D. *et al.*, *Nucleic Acids Research 12*:4539-4557 (1984)).

<u>Polymerase chain reaction (PCR) amplication</u>

The LKP4 *hip*P and LKP5 *hipP* pilin genes were amplied by PCR from NTHi strains P861249 and P860688 respectively, using standard PCR amplication protocols (Saiki, R.K. *et al.*, *Science 239*:487-491 (1988)).

<u>DNA sequencing</u>

The *hipP* gene contained on plasmid pPX551 and the entire LKP1 operon contained on plasmid pHF1 were sequenced with standard M13 sequencing primers and with overlapping sense and antisense primers. All the DNA sequencing was done on an Applied Biosystems (ABI) 373A DNA Sequencer, utilizing the *Taq* thermal cycling DyeDeoxy™ Terminator sequencing kit from ABI, part # 901497. The LKP4 and LKP5 serotypes were sequenced directly from the PCR products using the PCR amplication primers and internal synthetic primers based on the LKP1 sequencing study.

<u>SDS-PAGE analysis</u>

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed in a 70 by 100 mm mini-gel system (Bio-Rad, Richmond, CA) using the method of Laemmli (Laemmli, U.K., *Nature (London) 227*:680-685 (1970)). Samples were reduced with β-mercaptoethanol or DTT in sample preparation buffer and boiled for 5 min. Gels were run at 150 V constant voltage. Separated proteins were detected by staining with Coomassie brilliant blue G-250 (Sigma).

<u>Partial purication of pili</u>

LKP pili were puried according to previously described methods using differential pH solubility (Brinton, C.C., Jr. *et al.*, *Pediatr. Infect. Dis. J. 8 Suppl.*:54-61 (1989)). Briefly, piliated bacteria were

harvested from liquid culture by centrugation and washed 2X in phosphate buffered saline, pH 7.2. The bacterial pellet was resuspended in 100 mM tris, pH 10.3, containing 150 mM NaCl at a ratio of 4 ml buffer/g wet weight of cells. Pili were sheared off of the cells by blending in an Oster miniblender for three 3 min bursts at 4°C. Bacterial debris was separated by centrugation and discarded. The supernatant was dialyzed against 50 mM NaAcetate, pH 5.0 overnight to precipitate pili and denature other proteins. The pellet was collected by centrugation at 15,000 x g at 4°C and dissolved overnight in 50 ml of 0.01 M CAPS buffer, pH 10.4 with gentle rocking. This cycle of acid precipitation and solubilization in basic buffer was repeated two more times. The final acid pellet was then resolubilized in 0.01 M NaPhosphate, pH 10.4 and non soluble material discarded. This soluble fraction was referred to as partially puried pili.

Sequence of the LKP1 operon

The LKP1 operon was sequenced as described above and the full sequence is set forth in SEQ ID NO:4. Sequence analysis identied six potential open reading frames (ORFs) in the LKP operon, including the *hip*P (at about nucleotide 1882-2532 of SEQ ID NO:4) and *hip*C (at about nucleotide 2854-3630of SEQ ID NO:4) genes. All six ORFs in the LKP operon were identified as homologous to equivalent pilus operon genes in the pilus superfamily, as defined by multiple sequence alignment of proteins. Analysis of sequence alignment was also performed using Entrez Sequences Database Release 10.0 of the National Center for Biotechnology Information (National Library of Medicine, Bethesda, MD). Derived amino acid sequences of the ORFs are shown in Table 5 (SEQ ID NOs:5-10). A function for each reading frame was assigned based on sequence

alingment analysis. There are five ORFs which appear to be grouped into an operon controlled by the *hipC* promoter region. After the *hipC* (periplasmic chaperon) gene, the second reading frame *hipR* (at about nucleotide 4016-6238 of SEQ ID NO:4) was designated, a membrane anchor protein, the third ORF *hipM* (at about nucleotide 6259-6873 of SEQ ID NO:4) was designated, a tip associated protein, (also referred to herein as a minor tip protein) and the fourth ORF *hipA* (at about nucleotide 6955-8265 of SEQ ID NO:4) was designated, a tip adhesin protein. The pilin gene (*hipP*) and the periplasmic chaperon gene (*hipC*) are transcribed in opposite orientations as in the LKP 4 operon with the promoter region having the previously identied TA repeats(van Ham, S.M. *et al.*, *Cell 73*:1187-1196 (1993)). Since pHF1 expresses LKP1 pili in *E. coli*, there are 10 TA repeats in the intrapromoter region as described by van Ham *et al.*. These TA repeats are responsible for phase variation of the LKP pili phenotype, with loss of some of the repeats resulting in loss of piliation and a TA repeat number between 10 or 11 allowing expression of the LKP operon. As identiifed on the LKP1 operon was an ORF encoding an integrase (at about nucleotide 1495-1868 of SEQ ID NO:4). Also located on the LKP1 operon was a sequence encoding an enzyme, peptidase (at about nucleotide 8395-9347 of SEQ ID NO:4).

The predicted size of the LKP1 *hipP* gene product is approximately 21.2 kilodaltons, assuming a signal sequence length of 20 amino acids, while the observed molecular weight in SDS-PAGE gels is approximately 27 kilodaltons. Part of this may be explained by the anomalous sequence migration of LKP pilins in general in SDS-PAGE gels (mature LKP4 migrates at a molecular size of 24 kilodaltons while its predicted size is 22.1 kilodaltons) but the exact explanation remains unknown.

Sequence comparison of LKP serotypes 1, 4, and 5 *hipP* genes

This report represents the first sequence analysis of the *hipP* genes encoding LKP serotypes 1 and 5 (Figure 1). The *hipP* gene from an LKP4 expressing *Hib* strain has also been sequenced (van Ham, S.M. *et al.*, *EMBO Jour.* *8*:3535-3540 (1989)) and the derived amino acid sequence shows 99% identity with the LKP4 *hip*P derived amino acid sequence contained herein. The *hipP* gene sequences from *Hib* strains Eagan and M43 have been published (Forney, L.J. *et al.*, *Infect. Immun.* *59*:1991-1996 (1991)). The LKP1 *hipP* gene should encode a protein of approximately 21.5 kD while the predicted molecular weight of the LKP 4 *hipP* protein is 23.8 kD. The actual *hipP* gene products observed in recombinant *E. coli* are of approximately the correct sizes in Western blots for LKP4 and LKP5, but the LKP1 pilin runs aberrantly at a higher molecular weight than predicted at 26 kD. MacVector software was used to assess homology of these genes, with LKP4 *hipP* and LKP5 *hipP* proteins being 70 and 67% identical to LKP1 *hipP*, respectively. The alignment between the sequences is very good at the amino termini of the proteins, with three major areas of sequence divergence in the LKP1, 4, and 5 serotype genes farther into the proteins as shown in the Figure. Since little cross reactivity is observed between anti-LKP1, anti-LKP4, or anti-LKP5 sera with intact pili of a heterologous serotype, the sequences responsible for the serotype specicity of the typing antisera must be located in these regions. By comparison of the sequences in GenBank to the LKP4 sequence, the *H. influenzae* type b M43 pilin (Gilsdorf, J.R. *et al.*, *Infect. Immun.* *58*:1065-1072 (1990)) sequenced by Gilsdorf *et al.* also appears to be an LKP4 serotype gene (data not shown).

Example 2: Construction of LKP Type Pili-Producing *E. coli* Recombinants

Bacterial strains

Piliated *Hflu* strains used for *E. coli* recombinant construction are LKP11/CB59, LKP10/88-0807 and LKP12/88-0677. Hemagglutination and serum agglutination were examined before making genomic library. *E. coli* strains XL1-Blue ^{MR} and HB101 were used as cloning host cell.

DNA library construction and cosmid vector DNA

Chromosomal DNA from LKP11, LKP10 and LKP12 were extracted and puried respectively by standard techniques. *Hflu* genomic DNA size is about 1.8 x 10^6 bp. Chromosomal DNA was partially digested with restriction enzyme *Sau3A* I. Approximately 30kb DNA fragment was eluted from LMTA-gel (Sigma) and puried by phenol-chloroform method. The final DNA concentration is about 1ug/ul.

Vector DNA SuperCos I (Stratagene, La Jolla, CA) was digested with *Xba* I and dephosphorylated with calf intestinal alkaline phosphatase (CIAP). The *Xba* I and CIAP treated vector DNA was then digested with *Bam* HI restriction enzyme. About 6.5kb vector DNA fragment was obtained.

LKP11/CB59, LKP10/88-0807 and LKP12/88-0677 DNA fragments were ligated at the *Bam* HI site of the vector DNA SuperCos I, respectively. The ligated DNA was packaged into 1 phage particles using Ciga-pack Gold kit (Stratagene, La Jolla, CA). The host cell for packaging was XL1-Blue^{MR}.

Library screening

Recombinant expressed LKP type pili were screened by colony blot method. The concentration of anti-pilius sera from LKP11, LKP10 and LKP12 was 1:1000 dilution. The percentage of positive colony was 40/4200 for LKP11, 9/700

for LKP10 and 1/600 for LKP12. The cell piliation was examined by EM. The recombinants were veried by further HA and SA assay and they were named CLJ11 for LKP11, CLJ10 for LKP10 and CLJ12 for LKP12 (Figures 2A, 2B and 2C). Recombinants DNA was extracted and transformed to *E. coli* strain HB101 because XL1-Blue cell expresses type I pili. The recombinants DNA size is about 18.5kb for CLJ11. This was obtained by digestion and subsequent ligation using restriction site on insert and vector DNA. CLJ10 DNA is about 25kb and 35kb is for CLJ12. Partial DNA sequence is available for these recombinant inserts.

<u>Example 3: Protocols for the Purication of an LKP Pilus from an E. coli Recombinant Strain Using the Liquid Phase Method</u>

<u>General Protocol</u>

1. Inoculate recombinant *E. coli* cells in a 3 ml of LB media containing ampicillin and grow at 37°C until the OD 540nm reading reaches 0.6-0.8 (3-4 hours).
2. Transfer the cell suspension to 50 ml of medium and grow at 37°C until the reading at 540 nm reaches 0.8-1.0 (4-5 hours).
3. Transfer the 50 ml of cell suspension to 1L of medium in 2.8L flask and grow at 37°C overnight (16-18 hours) until a reading at 540 nm of 4.0-5.0 is obtained.
4. Harvest cells by centrugation at 5000 rpm for 15 minutes.
5. Resuspended the cells in 50 nM acetate buffer pH 5.0 and keep the suspension at room temperature for 1 hour.
6. Blend at 11000 rpm in large cup, or 14000 rpm in small cup, with omnimixer, ice for 3 minutes.
7. Titrate to pH 8.0 with 1 M Tris-HCI and let stand for 3 hours at room temperature.

8. Centruge at 12000 rpm for 20 minutes at 4°C. Weigh all pellets and discard.
9. Add 10 ul of DNase and RNase for each 100 ml of prep. Mix thoroughly and let stand for 10 minutes at room temperature.
10. Dialyze against several changes of 50 mM acetate buffer pH 5.0 overnight. Of the prep does not reach pH 5.0 overnight, then dialyze longer against more changes of buffer.
11. Centruge at 16000 rpm for 60 minutes at 4°C to pellet the protein precipitant and pilus crystals.
12. Resuspend the pellet in about 25% original volume with 25 mM Tris-HCI buffer pH 8.0.
13. With gentle stirring add Triton X-100 and EDTA to the prep to yield final concentration of 0.2% and 5 mM. Stir gently overnight at 4°C.
14. Clary the prep by centruging at 16000 rpm for 60 minutes at 4°C.
15. Add NaCl and PEG 8000 to final concentration of 0.5 M and 3.0% respectively then incubate and prep over ice for 2 hours.
16. Centruge the prep at 16000 rpm for 60 minutes at 4°C to pellet the pilus crystals.
17. Resuspend pellet in 25 mM Tris-HCI pH 8.0 in 1/3 of previous volume. Use less solution a lesser yield of pilus crystals is obtained.
18. Repeat steps 13 to 17.
19. Resuspend pellet in 25 mM Tris-HCI pH 8.0. Depending on purity and amount of material alternative solubilization and crystallization steps may be continued as needed.

During purication, sample after each step and use SDS-PAGE to examine purity of the samples. Dark field microscopy assay is needed in assistance for purity

checking. It is necessary to use UV scanning to determine any contamination by DNA or RNA.

Since Triton X-100 has a strong absorbance at 280 nm, it is important to remove the residual of Triton X-100 by crystallization, one time, or more, of pili by PEG and NaCI after purication. This avoids false reading at 280 nm when one determines concentration of pilus preparation by UV method.

Purification of LKP 5 Pili

1. Harvest in 80 mM PBS pH 5.0 using 5-10 ml/tray.
2. Titrate prep to pH 5.0 with 6N HCI if necessary.
3. Blend with omnimixer over ice for 3 minutes (average speed=9800 rpm) (up to 11000 rpm if possible in larger cups and up to 14000 rpm in small cups).
4. Titrate to pH 9.0 with 5 M NaOH and let stand for 3 hours at room temperature. It may be necessary to stir gently to prevent pH changes. Monitor pH throughout and adjust if needed. (If cultures were grown in broth, then titrate with a 1 M solution of buffer (Tris) instead of NaOH.)
5. Centrifuge at 15300 g for 20 minutes at 4°C. Transfer supernatant to clean bottles and clarify a second time as before. Weigh all pellets and discard.
6. Adjust pH of supernatant to 8.0 and add 10 ul of DNase and RNase for each 100 ml of prep. Mix thoroughly and let stand for 10 minutes at room temperature.
7. Dialyze against several changes of 40 mM acetate buffer pH 5.0 overnight. If prep does not reach pH 5.0 overnight then dialyze longer against more changes of buffer.

8. Centrifuge at 18600 g for 60 minutes at 4°C to pellet the pilus crystals (crystals not typical for clear pili).
9. Resuspend the pellet in about 25% original volume with 25 mM Tris-CHI pH 9.0 using rubber policeman. Stir gently at 4°C (avoid forming) several hours. Break up large pieces with gentle pipeting as needed.
10. With gentle stirring, add Triton X-100 (2% stock) to the prep to yield a final concentration of 0.4% and add EDTA (25 mM stock) to a final concentration of 5 mM. Incubate overnight at 4°C.
11. Clarify the prep by centrifuging at 186000 g for 60 minutes at 4°C. Transfer supernatant to clean flask.
12. Adjust the pH of the supernatant to below 8.0 using 1 N HCI.
13. Add NaCI (5 M stock) to a final concentration of 0.5 M and PEG (30% stock) to final concentration of 3% then incubate the prep over ice for 0.5 hour. Inspect in darkfield for crystals. Increase time if needed but it is critical not to overexpose pili to PAGE because resolubilization becomes increasingly difficult with increasing times.
14. Centrifuge prep at 18600 g for 60 minutes at 4°C to pellet the pilus crystals.
15. Wash pellet with 40 mM citrate buffer pH 5.0 to remove excess PEG/NaCI. Then centrifuge at 186000 g for 60 minutes (2 times).
16. Resuspend pellet in 25 mM Tris-CHI pH 9.0 in 1/3 to 1/2 previous volume. Solubilize by swirling followed by gentle pipetting. Run sample on a gel to check for purity. If necessary, continue with step 17.
17. Add Triton x-100 to the prep to yield a final concentration of 0.4% and add EDTA to a final concentration of 5 mM then incubate overnight at 4°C (see step 10 for details).

18. Adjust the pH of the prep to below 8.0 using HCI (between 7 and 8).
19. Add NaCI to a final concentration of 0.5 M and PEG to a final concentration of 3% then incubate the prep over ice for 0.5 hours (see step 13 for details).
20. Centrifuge prep at 186000 g for 60 minutes at 5°C to pellet pilus crystals.
21. Resuspend the pellet in 252 mM Tris-HCI pH 9.0 to solubilize pili (see step 16 for details). Check for purity by SDS-PAGE. If necessary, continue with step 22.
22. Add Triton X-100 to the prep to yield a final concentration of 0.4% and add EDTA to a final concentration of 5 mM then incubate overnight at 4°C (see step 10 for details).
23. Clarify by centrifuging at 18600 g for 60 minutes at 4°C.
24. Add NaCI to a final concentration of 0.5 M and PEG to a final concentration of 3# then incubate the prep over ice for 0.5 hour (see step 13 for details).
25. Centrifuge at 18600 g for 1 hour at 4°C. Discard supernatant.
26. Resuspend pellet in Tris-HCI pH 9.0. Depending on amount and purity of material, alternating solubilization/crystallization steps may be continued as needed.

During purification process, monitor pellet material and supernatant by darkfield and/or gel and/or scan. May need to reprocess

Purity by SDS-PAGE check: Repeat Triton step as needed, but avoid SDS reaction steps in previous protocols because of high losses of pili.

Example 4: Purication of LKP pili by HPLC and other Column Methods

Besides detergent extraction and PEG precipitation, LKP pili also can be puried by HPLC, FPLC and other column methods. These methods are good particularly for unknown LKP pili. Normally, pili are partially puried by extraction and precipitation first until the pilus solution is clear, concentrated and very small size. the preparation still is not pure as determined by SDS-PAGE, column methods would be the application of the choice. Sizing columns are preferred to be used for this purpose. Prior to loading to a column, treatment for further purication of the pilus sample is important. The detergent used for partial purication of pili should be removed from pilus samples by dialysis or other known techniques. Detergent signicantly reduces column separation resolution. Size exclusive column requires a small sample volume.

For HPLC or FPLC, the loading volume of 50 ul to 200 ul is recommended, and for other routine LC gel filtration columns, the sample loading volume depends on the length and size of the column. A 1 ml of pilus sample is preferred for a column with a total volume of 50 ml. Since pili have a low absorbance at 280 nm, a higher sensitivity for monitor is recommended. Available protein eluted from column can be monitored at 230 nm. Further purication of proteins can be performed by HPLC. Column methods of purification are also useful for isolation of pilin from pili.

Example:5 Protocol for the Purication of an LKP Pilus from an Hflu Strain or E. coli Recombinant Strain Using Solid Phase Method

Generally speaking, recombinant strain expresses pilus structural protein better than parent strain, H flu,

does, therefore, it is easier to pury pili from the recombinant cells. However, due to the fact that the *E. coli* recombinant strain expresses the pilus protein as same as the parent *Hflu* does, purication procedures of pilus rods from *Hflu* or from recombinant strain are basically the same. Growth of *Hflu* strain requires choclate agar media and certain CO_2 and humidity. Growth of *E. coli* recombinant strain needs LB agar media containing ampicillin.

1. Harvest in 80 mM PBS pH 5.0 using 5 ml/tray. Use a smoothed glass edge to scrape wet cells and then transfer the cell suspension to omnimixer cup. less cells are made surface only use media surface moisture to collect wet cells.
2. Titrate prep to pH 5.0 with 2 M acetate buffer necessary.
3. Blend at 14000 rpm with omnimixer over ice for 3-5 minutes.
4. Titrate to pH 8.0 with 1 M Tris-HCI buffer and monitor pH change by pH meter. It may titrate to pH with 2.5 or 5 M NaOH instead of Tris buffer, prep contains a lot of wet cells. Be careful to avoid lysis of cells when use NaOH. Incubate the prep at room temperature for 3 hours.
5. Centruge at 12000 rpm for 20-30 minutes at 4°C. Weigh all pellets and discard.
6. Add 10 ul of DNase and RNase for each 100 ml of prep. Mix thoroughly and let stand for 10 minutes at room temperature.
7. Dialyze against several changes of 50 mM acetate buffer, pH 5.0, overnight. If the prep does not reach pH 5.0 overnight, then dialyze longer against more changes of buffer.
8. Centruge at 16000 rpm for 60 minutes at 4°C to pellet protein precipitate and pilus crystals.

9. Resuspend pellet in about 25% original volume with 25 mM Tris-HCI buffer, pH 8.0.
10. With gentle stirring, add Triton X-100 and EDTA to prep to yield final concentration of 0.2% and 5 mM. Stir gently overnight at 4°C.
11. Clary prep by centrugation at 16000 rpm for 60 minutes at 4°C.
12. Add NaCl and PEG 8000 to final concentration of 0.5 M and 3.0%, respectively, then incubate the prep over ice for 2 hours. LKP pili with different length and dimer may be crystallized in different concentrations of NaCl and PEG 8000. Therefore a concentration test for NaCl and PEG to crystalize different pili is important.
13. Centruge at 16000 rpm for 60 minutes at 4°C to pellet pilus crystals.
14. Resuspend pellet in 25 mM Tris-HCI, pH 8.0 in 1/3 previous. Use even less solution a smaller yield of pilus crystal is found.
15. Repeat from step 10 to step 14.
16. Resuspend pellet in 25 mM Tris-HCI, pH 8.0. Depending on purity and amount of material, alternate solubilization and crystallization steps may be continued as needed.

During purication, sample after each step and use SDS-PAGE to examine purity of the samples. Dark field microscopy assay is needed in assistance for purity checking. It is necessary to use UV scanning for finding out any contamination by DNA or RNA.

Since Triton X-100 has a strong absorbance at 280 nm it is wise to remove the residual of the detergent by one more time crystallization of pili by PEG and NaCI after purication. This avoids false readings at 280 nm when one determines concentration of pilus preparation by UV method.

Example 6: Construction of MBP-Δ3'Tip Fusion Protein

The genetic fusion was constructed by using PCR primers to obtain a portion of the LKP1 tip gene from pHF1 which would be in frame with the MBP protein gene in the vector pMAL-p2. The primers were designed so that the carboxyl terminal of approximately 100 amino acids of the tip protein would be deleted and replaced with a stop codon. The amino terminal portion of the protein was PRC'd in frame with an appropriate restriction site at the approximate point of the signal sequence cleavage site which was determined by analogy to other bacterial signal sequences and the hydrophobicity profile of the deduced amino acid sequence of the tip protein. The amino acid sequence of the fusion protein is shown in Figure 2. The partial sequence of the LKP tip protein of the fusion protein is underlined.

Expression of the fusion, purication, and antisera production

The protein was expressed in *E. coli* BL21 (an *onnipT.lon* K-12 strain) grown in SOB broth containing ampicillin at 100 µg/ml at 28 C after induction with 0.2 mM IPTG. The cells were pelleted by centrugation and washed 1 time in PBS. The cells were resuspended in 20 mM Tris, pH 7.5 containing 2 mM EDTA and 400 mM NaCl at a ratio of 20 ml/liter of original culture. The cells were lysed by passing through a French pressure cell 3 times and the cell debris removed by low speed centrugation at 8 times x g for 20 minutes at 4°C. The supernatant was diluted 5-fold in the same buffer used for breakage and passed over a 15 ml bed volume amylose resin column at 1 ml/min at room temperature. After the lysate was run over the column, the column was washed with 15 bed volumes of the lysing buffer at 5 ml/min. The bound material was eluted using washing buffer containing 10 mM maltose. The

elution was done with 50 ml of buffer at 1 ml/min and the eluant pooled. The resulting protein mixture was analyzed by SDS-PAGE and Western Blot and anti-MBP sera and found to contain the fusion, breakdown products, and full length MBP. Little other material was detected.

The fusion proteins, MBP and breakdown products eluted as a complex. Mice were immunized with 10 μg doses of the complex using 100 μg MPL as adjuvant. Immunizations were done subcutaneously at weeks 0, 4, and 6 and the mice exsanguinated on week 8. The negative control sera was mouse anti-MBP sera made against puried MBP using the same purication and immunization protocols.

<u>Anti-GST sera</u>

The GST fusion was constructed using the complete LKP tip gene, including the signal sequence. The gene was PCR'd out from PHF1 with the appropriate restriction enzyme sites for insertion into pGEX-3X in frame, and expressed in *E. coli* DH5α. The cells were grown in SOB containing 100 μg/ml ampicillin and induced with IPTG at 0.2 mM at 37°C for 2 hours. The cells were harvested and washed in PBS, then resuspended in PBS and lysed by passing through a French pressure cell. Cell debris was harvested by centrugation, and washed 3 times with buffer containing 1% Triton X-Zwittergent 3-14 and the inclusion bodies recovered by centrugation. The inclusion bodies were solubilized in 5 M guanidine HCl and analyzed by SDS-PAGE. The guanidine concentration was lowered to 2.5 M by dialysis and the soluble inclusion bodies stored at 4°C. The antisera was made by running preparative 10% SDS-PAGE gels and cutting the fusion band out of the gel. The acrylamide-protein band was minced using a scalpel and mixed with MPL (100 μg) and injected into mice 3 times at weeks 0, 4, and 6. Mice were bled at week 8.

Example 7: Removal, Purication and Identication of H. influenzae LKP Pilus Tip Adhesin Protein

This is the first demonstration that tip adhesin protein from *H. influenzae* LKP1 pili can be removed without depolymerization of pilus rods. Free tip adhesin protein can be isolated and puried by means of dialysis and prep-electrophoresis. Puried tip adhesin can be identied by the antiserum from a constructed genetic fusion protein, which is from a portion of LKP1 tip gene and MBP (maltose binding protein) gene, using Western blot analysis. Specic binding was detected between the puried tip protein and fusion protein antiserum, which clearly shows that the protein puried from LKP1 pilus prep is LKP1 tip adhesin protein.

Activity assays with human red blood cell (RBC) ghosts demonstrated that puried tip protein binds to a native ghosts preparation but not does not bind to denatured RBC ghosts, indicating that puried tip protein is biologically functional or at least partially functional.

Removal of Tip Protein from Pilus Rods

1. Dialyze puried LKP1 pili in 200 mM Gly-HCl buffer, pH 2.0 containing 5 M NaCl, at room temperature for 4 to 6 hours.
2. Transfer the dialysis bag into a 25 mM Tris-HCl buffer, pH 8.0 and dialyze for several hours till the pH of pilus prep reaches to pH 8.0.
3. Add SDS to the pilus prep to a final concentration of 0.1% and incubate in 4°C for 10 hours.
4. Dialyze the pilus prep in 50 mM citrate buffer, pH 5.0 overnight.
5. Pilus aggregates can be removed by centrugation and most free tip protein is retained in the supernatant.

Tip protein can be completely removed by 2% SDS in 25 mM Tris buffer without depolymerization of pilus rods, but the SDS may damage the activity of the protein. 0.1% SDS only removes about 20-30% of total tip protein, however, the protein maintains biological activity. The results also demonstrated that 4M urea and 2M GuHCl in pH 2.0 buffer can partially remove tip protein from pilus rods without depolymerization.

Purication of tip protein

1. Mix concentrated tip protein with SDS-PAGE sample treatment buffer without the SDS and β-mercaptolethanol. The ratio is 2.5 ml of pilus prep to 0.3 ml of sample treatment buffer.
2. Load the sample to a 12% SDS-PAGE (0.1% SDS) in Prep-Cell (Bio-Rad) with the length of stacking gel of 0.8-1.0 cm and running gel of 5cm.
3. Run the gel at 300 volt with cooling system for 6-8 hours, and monitor the elution at 280 nm.
4. Pool the fractions containing tip protein and concentrate.
5. Determine the purity of the pooled fractions by mini-SDS-PAGE. The identication of puried tip protein by anti-KLP1-MBP fusion protein is shown in Figure 4. The binding activity of puried tip protein with human red cell ghosts is shown in Figures 5 and 6. Figure 7 compares adhesin proteins from different LKP type pili by SDS/PAGE.

Example 8: Serotype Analysis

The *Haemophilus influenzae* (*Hflu*) bacterioplex is a differentiated complex of bacterial phases, or cell types, socially organized to facilitate the protein appendages expressed on the surface of *Hflu*, and also secreted from *Hflu* in free form, carrying specic adhesion determinants

for binding to human cell membrane receptors. Pili adapt pathogenic bacteria to le in vertebrate hosts by mimicking the functions of the host's own proteins. Pilus functions include attaching bacteria to a variety of host cells and tissues and stimulating the host's immune system in ways which benefit the bacteria and damage the host. Pili are transmission, virulence, dissemination, pathogenicity and immunity factors in most bacterial diseases.

The expression of pili is controlled by a genetic switching mechanism, phase variation, in which pilus expression and pilus type are switched on and off at probabilities which vary with and are determined by conditions and signals in the immediate environment of the bacteria. Under some conditions the switching probabilities can be very high, as high as 10^{-2} per bacterial cell division. Under other environmental conditions the probability of the same phase switch can be 10^{-6} or lower. Phase switching is accompanied by both reversible and irreversible rearrangements in the DNA of pilus operons. Phase switching during *in vitro* growth is frequently accompanied by deletions to pilus operon genes such that nonpiliated phases remain irreversibly in that phase.

By purying *Hflu* pili from different isolates and producing antisera to the puried preparations distinct LKP pilus serotypes have so far been identied. The expression of the different serotypes is used as a marker to identy the different piliation phases of the *Hflu* bacterioplex.

TABLE 1

		L=1	L=2	L=3	D=3	D=4
LKP1	N=4	0	0	4	0	4
LKP2	N-2	0	1	1	0	2
LKP3	N=0	0	0	0	0	0
LKP4	N=1	0	1	0	0	1
LKP5	N=5	0	1	4	0	5
LKP6	N=12	0	2	8	1	9
LKP7	N=3	0	0	2	0	2
LKP8	N=0	0	0	0	0	0
LKP9	N=0	0	0	0	0	0
LKP10	N=26	1	8	17	2	24
LKP11	N=22	0	6	16	0	22
LKP12	N=12	0	3	7	2	8
LKP13	N=0	0	0	0	0	0
LKP14	N=9	1	2	6	1	8
LKP15	N=6	0	5	1	0	6
LKP16	N=9	0	4	5	3	6
LKP17	N=17	0	6	11	2	15
LKP18	N=12	1	4	7	1	11
LKP19	N=3	0	1	3	0	3
LKP20	N=15	1	6	8	3	12
Total Strains = 77		4	50	99	15	136

L=1 is length <0.2μ
L=2 is length <0.2μ<0.5μ
L=3 is length <0.5μ
D=3 is 3nm diameter ("thin")
D=4 is 4nm diameter ("thick")

The frequency of each LKP serotype was determined for all serotypable cultures and for all cultures expressing

by counting types on both single expressors and multiple expressors. Sixteen of the 20 serotypes were found on typically LKP piliated cultures and 90% of these cultures were serotypable in the 20-type system. The frequency distribution of serotypes for these cultures is shown in Table 1.

Three different LKP pilus operon genes were selected, the pilin gene, anchor gene and adhesin gene, which had all exhibited sequence similarity among different serotypes in multiple sequence alignments, but were also characteristic of *Hflu* LKP pili. Sequences were selected from these genes that would serve as suitable primer sequences flanking each gene for use in a PCR reaction.

LKP1 Pilin: HF2 5'>AGCTGGATCCTTGTAGGGTGGGCGTAAGCC<3'
(SEQ ID NO:16)
HF1 5'> AACGGATTCGTTTGCTGTTTATTAAGCCTT<3'
(SEQ ID NO:17)

LKP1 Anchor: R5 5'>GCCGCACCTTTGATGAACG>3'
(SEQ ID NO:18)
R3 5'>GGCAAATACGCACCGCTAAAT>3'
(SEQ ID NO:19)

LKP1 Adhesin: A5 5'>CGGACGAAGATGGTACAACGA>3'
(SEQ ID NO:20)
A31 5'>CCAAGCTTGGCCCGACATTATTATTGATATGACA>3'
(SEQ ID NO:21)

All three pairs of primers were synthesized and used in a PCR reaction to amply segments of DNA extracted from *Hflu* isolates.

Data showing the presence of LKP pilus operons in tested *Haemophilus influenzae* strains is shown in Table 2.

TABLE 2						
CORRELATION BETWEEN THE PRESENCE OF LKP PILUS OPERON GENETIC MATERIAL IN *H. INFLUENZAE* ISOLATES AND THE EXPRESSED LKP PARAMETERS OF PILIATION AND HEMAGGLUTINATION						
LKP Parameter	Total	PCR Done	PCR +	PCR -	Fraction PCR +	Percent PCR +
Pilus Length 0	74	68	59	9	59/68	87%
Pilus length 3	101	93	82	11	82/93	88%
HA+	148	139	115	24	115/139	83%
HA-	166	149	119	30	119/149	80%
Pilus Diam. 3	40	38	28	10	28/38	74%
Pilus diam. 4	172	159	136	23	136/159	86%
Serotypable	189	173	149	24	149/173	86%
Not serotypable	54	63	53	10	53/63	84%

1. Pilus length 0 means nonpiliated.
2. length 3 means >0.5 microns (longest, typical of LKP pili).
3. HA+ means positive for hemagglutination of human red blood cells; typical of LKP pili. (These isolates are not recalcitrant by definition.)
4. HA- means negative for hemagglutination of human red blood cells; typical of SNN pili. (These isolates are recalcitrant since all isolates were hemadsorbed at least once.)
5. Pilus diameter 3 means the isolates express pili with diameters typical of SNN pili.
6. Pilus diameter 4 means the isolates express pili with diameters typical of LKP pili.
7. Serotypable means the isolates agglutinate under standard conditions with at least one of the LKP pilus typing antisera in the 1-20 system.
8. Not serotypable means the isolates do not agglutinate with any of the LKP pilus typing antisera in the 1-20 system.

Example 9: Hybridization Assay for Haemophilus Influenzae

Assay Probe Construction

An approximately 1100 bp fragment from plasmid pHF1 (Karasic, R. *et al.*, *Pediatr. Infect. Dis. J. 8 (Suppl.)*:S62-65 (1988)) which contains the LKP1 serotype operon was amplied by PCR using primers which hybridize at the 5′ and 3′ ends of the *hip*A gene. This gene encodes the tip adhesin protein of the LKP1 pili. The PCR reaction included digoxigenin labeled dUTP along with the

four dNTPs to label the PCR reaction product with digoxigenin. This probe was electrophoresed on an agarose gel and puried by cutting out the ~1.2 kb band and extracting the DNA by standard methods. The probe was redissolved in 30 μl of appropriate buffer.

Hybridization Assay

Eleven randomly chosen Haemophilus influenzae clinical isolates were grown on BHI-XV plates at 37°C with 5% CO_2 and also streaked onto BHI agar. All isolates grew only on the BHI-XV plate, indicating that they were *H. influenzae*. The isolates included 2 *Hib* strains and 9 *NTHi*. The strains were inoculated onto a nylon membrane placed onto BHI-XV agar. Five clinical isolates of another respiratory pathogen, *Moraxella catarrhalis* were also spotted onto the filter. The bacteria were grown overnight at 37°C in 5% CO_2. After growth, 2 *Bordetella pertussis* strains were spotted onto the filter. Filters were processed for colony hybridization according to the method of Maniatis *et al.* (*Molecular Cloning: A Laboratory Manual*, 1991, Cold Spring Harbor Laboratories, Cold Spring Harbor, NY). Filters were blocked in pre-hybridization solution as described by Boehringer-Mannheim for the Genius™ system at 65°C for 3 hours. Colony debris was removed by gentle rubbing with wet paper towels. The probe, 30 μl, was added to 5 ml of pre-hybridization solution and boiled for 10 minutes to denature the DNA. Probe was immediately added to the filter and allowed to hybridize overnight at 65°C. Filters were washed in 2X SSC, 0.1% SDS, 2X for 5 min/wash at room temperature followed by 2, 15 minute washes with 0.2X SSC, 0.1% SDS at 65°C. Bound probe was detected using alkaline phosphatase labeled anti-digoxigenin antibodies as described by the manufacturer. Results are shown in Table 3.

Table 3 HYBRIDIZATION OF dig-LABELED LKP 1 TIP PROBE TO RANDOM CLINICAL ISOLATES

Bacterial Strain	Number of Positive Results: Strong Signal	Weak Signal	No Signal	# Total
H. influenzae	4	4	3	11
M. catarrhalis	0	0	5	5
B. pertussis	0	0	0	2

The probe was specic for *H. influenzae* with no hybridization seen with either *M. catarrhalis* or *B. pertussis*.

<u>Hybridization Assay of Nontypable Strains of Haemophilus influenza pili</u>

Ten LKP pili expressing NTHi strains which express differing serotypes of LKP pili, along with *Hib* Eagan were grown on a nylon filter overlayed onto chocolate agar at 37°C in 5% CO_2. An additional *NTHi* isolate was also included. After growth, two strains appeared yellow on the filter which was suggestive of non-*Haemophilus* bacteria, so they were tested by growth on BHI and BHI-XV. This experiment showed them to be contaminants and not *NTHi*. The filter was removed from the agar and processed as described above. The probe from the first experiment was reboiled and added to the filter as before, except that the hybridization temperature was lowered to 62°C. The filter was washed as before except that the wash temperature was also 62°C. Bound probe was detected as above. Results are shown in Table 4.

Table 4. HYBRIDIZATION OF dig-LABELED TKP TIP PROBE TO LKP TYPE STRAINS

LKP Serotype	Signal with probe	No signal with probe	ID of strain
5	Strong		NTHi
2	Moderate		NTHi
9	Strong		NTHi
1	Strong		NTHi
6	Moderate		NTHi
13	Strong		NTHi
4	Strong		NTHi
7	Moderate		NTHi
		X	Contaminant
		X	Contaminant
10	Weak		NTHi
4	Strong		Hib

The results set forth above establish that the DNA probes hybridized selectively to Haemophilus influenzae.

Equivalents

Those skilled in the art will recognize, or be able to ascertain using not more than routine experimentation, many equivalents to the specic embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

TABLE 5

AAGCTTGCATGCCTGCAGGTCGACTCTAGAGGATCATTCCATTGTGTTTTATCTTTTAATAAACACCAAGGT
GAGGTAGAAATATTCAGTTCATCAAGCAAGGATTTTTGCGTAAAACGATCGGCTAATAATCCAAATACATGT
TGATTAACGAAGTTTTTATGATTGCTGAGTAATTCAGTCAAAGGCGTTTTTTCCCAGCGTTCAATTTCCGCC
GTGATGATCGCATTTTCAGGTAAGTCAAAAACTGGCGCATTGAAGGCTAAGGGTTCAACATAAATATCTAAA
GGTGCACCAGCGTAACCTAACATTCTGCCGAGTTGTCCGTTGCCGAGAACATAAACGGTTGGGTATAAGGTG
GAGTTTTGCATAATATTTCTCGTTAAATTTACGAAAAAACAACCGCACTTTAAAAGTGCGGTCAGATCTGAA
GATATTTTTATGTGCGTGGATCGGGATTGTCCAGTACAGCACGAGTTTGGCTTTCACGGAAAGATTGCAAGC
GTGAAAGCAATTCTGCATCCCAACCTGCTAGAATTTGGGCTGCTAACAACCCAGCATTTGCCGCGCCTGCAG
AGCCAATCGCTAATGTTCCGACTGGAATCCCTTTTGGCATTTGCACAATTGAATAAAGGCTATCCACACCAC
TTAACATAGAACTTTTTACTGGCACCCCCAGCACTGGCACAAGTGTTTTGGCTGCGATCATACCAGGTAAAT
GTGCCGCACCGCCTGCACCAGCAATAATTACTTTATAGCCATTTTTTTGTGCATTTTCGGCAAATTCGAAAA
GTTTATCAGGCGTACGATGGGCAGAGACGACTTCCACATGATAAGGCACGTTTAATTCATCTAAAATCTGAG
TTGCCTCTTGCATAGTAGCCCAATCACTTTTTGACCCCATCACAACGGCAATTTGTGCAGTTTTTGACATGC
TATTTTCTCAATTTTCTAATTAAAAACGTGGTGTAGAATAGCATAGATTACATATATCGAGCAAACGTTTGC
TATTTATGTACGTATTAATGGGGATTATTTTATAATTATTTGATTTTTAAATTTTAGTAACTATACTTGATA
CCAAATTAATGGGCGATAGTTTATATGGGACGAACTGAAAAATTATTAGATAAGCTCGCACAATCAAAATCT
ACATTTAATTGGAATGAATTAGTTTCTTTGTTAGCTCAACAAGGTTATGAAAAGCGAGAAATGGCAGGTTCT
CGAGTGAGATTTTATAATAGAACACTCGAACATATGATTTTGTTACACAAGCCTCATCCTGAAAATTATATT
AAAGGCGGTGTTTTAAAGTCAGTGAAAGAATCATTAAAACAGGTAGGTATTCTATGAAGTTATTAAATTATA
AAGGTTATGTTGGCACGATTGAGGCGGATTTAGAAAACAATATATTATTTGGCAAACTTGCTTACATTCGTG
ATTTAGTGACTTACGAAGCAGAGTCATTATCTGAGTTAGAAAAAGAATTTCATCAATCTGTTGATTTATATT
TACAAGATTGTTTGGAATTAGGTAAAGAACCGAATAAGCCTTTTAAAGGTGTATTTAATGTACGAATTGGCG
AGGAATTGCATAGAGAAGCAACGATCATAGCTGGCGATCGTTCTCTTAATGCTTTTGTGACGGAAGCAATTA
AAGAAAAAATTTTTCGTGAAAAACCAAGTTTAAGATAACAAAACGTATTTACATTTTTTTTCATCACGTAGG
CTGGGCGTAAGCCCATGTAGAGACACATAAAAAAGATTTGTAGGCTAGGCGTAAGCTCACGTGGATACATAT
AAAAAAGATTTGTAGGGTGGGCGTAAGCCCACGCAGGATATAACAAACACGTGGGCTTAGATTGCATTACAT
TAGGAATTATTCGTAAGCAATTTGGAAATCAACTGAGGATTCTACTTTACCAGCTTCCGCTTGAGCTGTTGC
◄ GluTyrAlaIleGlnPheAspValSerSerGluValLysGlyAlaGluAlaGlnAlaThrAla
ATAGTATCTAGCGATATAGTGTAATTTCACATTGTTTTCACCGTTAATTGTAGCTTTTCCTGAAATATGATT
◄ TyrTyrArgAlaIleTyrHisLeuLysValAsnAsnGluGlyAsnIleThrAlaLysGlySerIleHisAsn
TTTATTCACAGTTTGTTGTGTTGCAACGTCATTTGTATTGCTATGCGTAAAATCTGTTGTTCCGTTGCCGAC
◄ LysAsnValThrGlnGlnThrAlaValAspAsnThrAsnSerHisThrPheAspThrThrGlyAsnGlyVal
AACTTCAATTGCATCTGTACCATTAGCATCAAAAAGCTGGATATTAACATTCTGTGCAGCATCATTTCCTGA
◄ ValGluIleAlaAspThrGlyAsnAlaAspPheLeuGlnIleAsnValAsnGlnAlaAlaAspAsnGlySer
TTTTGTATTTTTTAATGTATATTCATTATTTTCATCTGCATTTTTCCAAGAATAGAAATAAGCTCCAACTTT
◄ LysThrAsnLysLeuThrTyrGluAsnAsnGluAspAlaAsnLysTrpSerTyrPheTyrAlaGlyValLys
TGTTGCAACAGGCTTATTATTAGTAGTAGTAGTAGTAGAACAATTTTCTAAATTAATTGTAAATGGTGTTGG
◄ ThrAlaValProLysAsnAsnThrThrThrThrThrSerCysAsnGluLeuAsnIleThrPheProThrPro

TABLE 5 (cont.)

CATCGCTGTATCTTTTTTAGTTTTTAAATGATTTTTACCCACATCATTTAATACTACGCTCATATTTTTACT
◄ MetAlaThrAspLysLysThrLysLeuHisAsnLysGlyValAspAsnLeuValValSerMetAsnLysSer
ATCCGTTTTCACTTTACAAGTATTCTCAACAACCTTACCAAAGAAAGTAACTTTACCAGATGTTTCAGTACT
◄ AspThrLysValLysCysThrAsnGluValValLysGlyPhePheThrValLysGlySerThrGluThrSer
TACTTGAGGATCAGCAGCATTCGTTGCAAATGCCAATAAAATTAAGCTACCAAGAAGTGTTTTTTTCATAAT
◄ ValGlnProAspAlaAlaAsnThrAlaPheAlaLeuLeuIleLeuSerGlyLeuLeuThrLysLysMetIle
AAATTGCTCCATAAAGAGGTTTGTGCCTTATAAATAAGGCAATAAAGATTAATATAAACCGTTTATTAAAAT
◄ PheGlnGluMet
GCCAAAGGCTTAATAAACAGCAAACTTTGTTTTCCCAAAAAAAGTAAAAAACTCTTCCATTATATATATATA
TATATATAATTAAAGCCCTTTTTGAAAAATTTCATATTTTTTTGAATTAATTCGCTGTAGGTTGGGTTTTTG
CCCACATGGAGACATATAAAAAAGATTTGTAGGGTGGGCGTAAGCCCACGCGGAACATCATCAAACAACTGT
AATGTTGTATTAGGCACGGTGGGCTTATGCCTCGCCTACGGGGAAATGAATAAGGATAAATATGGGCTTAGC
► MetAsnLysAspLysTyrGlyLeuSer
CCAGTTTATGGATTTAATTATGTTGAAATGGGGAAAACAATGTTTAAAAAAACACTTTTATTTTTTACCGCA
► ProValTyrGlyPheAsnTyrValGluMetGlyLysThrMetPheLysLysThrLeuLeuPhePheThrAla
CTATTTTTTGCCGCACTTTGTGCATTTTCAGCCAATGCAGATGTGATTATCACTGGCACCAGAGTGATTTAT
► LeuPhePheAlaAlaLeuCysAlaPheSerAlaAsnAlaAspValIleIleThrGlyThrArgValIleTyr
CCCGCTGGGCAAAAAAATGTTATCGTGAAGTTAGAAAACAATGATGATTCGGCAGCATTGGTGCAAGCCTGG
► ProAlaGlyGlnLysAsnValIleValLysLeuGluAsnAsnAspAspSerAlaAlaLeuValGlnAlaTrp
ATTGATAATGGCAATCCAAATGCCGATCCAAAATACACCAAAACCCCTTTTGTGATTACCCCGCCTGTTGCT
► IleAspAsnGlyAsnProAsnAlaAspProLysTyrThrLysThrProPheValIleThrProProValAla
CGAGTGGAAGCGAAATCAGGGCAAAGTTTGCGGATTACGTTCACAGGCAGCGAGCCTTTACCTGATGATCGC
► ArgValGluAlaLysSerGlyGlnSerLeuArgIleThrPheThrGlySerGluProLeuProAspAspArg
GAAAGCCTCTTTTATTTTAATTTGTTAGATATTCCGCCGAAACCTGATGCGGCATTTCTGGCAAAACACGGC
► GluSerLeuPheTyrPheAsnLeuLeuAspIleProProLysProAspAlaAlaPheLeuAlaLysHisGly
AGCTTTATGCAAATTGCCATTCGCTCACGTTTGAAGTTGTTTTATCGCCCTGCGAAACTCTCGATGGATTCT
► SerPheMetGlnIleAlaIleArgSerArgLeuLysLeuPheTyrArgProAlaLysLeuSerMetAspSer
CGTGATGCAATGAAAAAAGTAGTGTTTAAAGCCACACCTGAAGGGGTGTTGGTGGATAATCAAACCCCTTAT
► ArgAspAlaMetLysLysValValPheLysAlaThrProGluGlyValLeuValAspAsnGlnThrProTyr
TATATGAACTACATTGGTTTGTTACATCAAAATAAACCTGCGAAAAATGTCAAAATGGTTGCCCCTTTTTCT
► TyrMetAsnTyrIleGlyLeuLeuHisGlnAsnLysProAlaLysAsnValLysMetValAlaProPheSer
CAAGCGGTATTTGAAGCCAAAGGCGTGCGTTCTGGCGATAAATTGAAATGGGTATTGGTTAATGATTACGGT
► GlnAlaValPheGluAlaLysGlyValArgSerGlyAspLysLeuLysTrpValLeuValAsnAspTyrGly
GCCGACCAAGAAGGCGAAGCCATCGCTCAATAATAGCGAACTAGTGTAGGGTGGGCTTTAGACCACCGATTA
► AlaAspGlnGluGlyGluAlaIleAlaGln
ACCATAACAAAGGTGGGCTGAAGCCCACCCTACAACCACAAAGAACGATTAATCTGTGAAAACAAAAATTTT
TCCCTTAAATAAAATTGCGTTTGCTTGTTCACTGCTATTGGCAAATCCTTTAGCGTGGGCGGGAGATCAATT
TGATGCCTCTCTTTGGGGAGATGGTTCGGTGTTGGGCGTTGATTTTGCCCGATTTAATGTAAAAAATGCCGT
GTTACCAGGGCGTTATGAAGCTCAAATCTATGTGAAATTTGAAGAAAAAGGCGTAAGCGATATTATTTTTGC

TABLE (cont.)

TGATAATCCTGCCACAGGTCGGACAGAATTATGCTTTACGCCTAAACTTCAAGAAATGCTGGATTTGATGGA
▶ MetLeuAspLeuMetAs
TGAAGCCATTGTGAAATCGCCCAATTCAGAAGATGACACTTGTGTCTTTGCTTCTGATGCTATTCCTAAAGG
▶ pGluAlaIleValLysSerProAsnSerGluAspAspThrCysValPheAlaSerAspAlaIleProLysGl
CACGTTTGAATATCAAAGCGGCGAAATGAAATTGAAACTTGAGCTCCCTCAAGCTCTCACTATTCGCCGACC
▶ yThrPheGluTyrGlnSerGlyGluMetLysLeuLysLeuGluLeuProGlnAlaLeuThrIleArgArgPr
AAGAGGCTATATTGCGCCATCTCGCTGGCAAACTGGCACCAATGCCGCTTTTGCAAATTACGATATCAACTA
▶ oArgGlyTyrIleAlaProSerArgTrpGlnThrGlyThrAsnAlaAlaPheAlaAsnTyrAspIleAsnTy
TTATCGTTCTGGTAATCCCGAAGTAAAATCCGAAAGTTTGTATGTGGGCTTGCGTAGTGGCGTAAATTTTGG
▶ rTyrArgSerGlyAsnProGluValLysSerGluSerLeuTyrValGlyLeuArgSerGlyValAsnPheGl
CAACTGGGCATTGCGTCATAGCGGCAGTTTTAGCCGTTTTGAAAACCAAAGTAGCTCGGGTTTTACTGATAA
▶ yAsnTrpAlaLeuArgHisSerGlySerPheSerArgPheGluAsnGlnSerSerSerGlyPheThrAspLy
GGGCAAAAATCATTACGAACGTGGCGATACCTATTTACAACGAGATTTCGCCCTGCTTCGTGGCAATGTCAC
▶ sGlyLysAsnHisTyrGluArgGlyAspThrTyrLeuGlnArgAspPheAlaLeuLeuArgGlyAsnValTh
TGTTGGGGATTTTTTCAGCACTGCCCGCATTGGCGAAAATTTTGGTATGCGTGGTTTGCGTATTGCCTCTGA
▶ rValGlyAspPhePheSerThrAlaArgIleGlyGluAsnPheGlyMetArgGlyLeuArgIleAlaSerAs
TGATAGAATGCTTGCCCCATCACAACGTGGTTTTGCCCCAGTGGTGCGTGGCGTGGCAAACACAAACGCCAA
▶ pAspArgMetLeuAlaProSerGlnArgGlyPheAlaProValValArgGlyValAlaAsnThrAsnAlaLy
AGTCAGCATCAAACAAAATGGCTATACGATTTATCAAATCACCGTTCCCGCAGGGCCTTTCGTGATTAACGA
▶ sValSerIleLysGlnAsnGlyTyrThrIleTyrGlnIleThrValProAlaGlyProPheValIleAsnAs
TTTGTATGCCAGCGGTTATAGCGGCGATTTAACGGTGGAAATCCAAGAAAGTGATGGTAAAGTGCGGTCATT
▶ pLeuTyrAlaSerGlyTyrSerGlyAspLeuThrValGluIleGlnGluSerAspGlyLysValArgSerPh
TATTGTGCCGTTTTCTAATCTTGCCCCGTTAATGCGTGTGGGGCATTTGCGTTATCAATTAGCTGGCGGACG
▶ eIleValProPheSerAsnLeuAlaProLeuMetArgValGlyHisLeuArgTyrGlnLeuAlaGlyGlyAr
TTATCGAATTGACAGCCGCACCTTTGATGAACGTGTGTTACAAGGCGTGTTGCAATATGGTTTAACTAATCA
▶ gTyrArgIleAspSerArgThrPheAspGluArgValLeuGlnGlyValLeuGlnTyrGlyLeuThrAsnHi
TCTCACGCTGAATTCAAGCCTGCTTTATACACGTCATTATCGTGCAGGGCTGTTTGGTTTTGGTTTAAATAC
▶ sLeuThrLeuAsnSerSerLeuLeuTyrThrArgHisTyrArgAlaGlyLeuPheGlyPheGlyLeuAsnTh

GCCGATTGGGGCGTTTTCTGCTGATGCCACTTGGTCGCACGCTGAATTTCCGCTAAAACATGTGAGCAAAAA
▶ rProIleGlyAlaPheSerAlaAspAlaThrTrpSerHisAlaGluPheProLeuLysHisValSerLysAs
CGGCTACAGCTTGCACGGCAGTTATAGTATTAACTTCAATGAAAGTGGCACCAATATCACGTTGGCAGCCTA
▶ nGlyTyrSerLeuHisGlySerTyrSerIleAsnPheAsnGluSerGlyThrAsnIleThrLeuAlaAlaTy
TCGCTATTCTTCACGGGATTTTTACACCTTAAGCGACACCATTGGTCTTAACCGCACTTTCAGACAATTTAG
▶ rArgTyrSerSerArgAspPheTyrThrLeuSerAspThrIleGlyLeuAsnArgThrPheArgGlnPheSe
CGGTGCGTATTTGCCTGAAATTTACCGCCCAAAAAATCAGTTTCAAGTGAGTTTAAGCCAAAGTCTGGGGAA
▶ rGlyAlaTyrLeuProGluIleTyrArgProLysAsnGlnPheGlnValSerLeuSerGlnSerLeuGlyAs
TTGGGGAAATCTCTATCTTTCAGGACAAACCTATAATTATTGGGAAAAACGTGGCACGAATACGCAATATCA
▶ nTrpGlyAsnLeuTyrLeuSerGlyGlnThrTyrAsnTyrTrpGluLysArgGlyThrAsnThrGlnTyrGl

TABLE 5 (cont.)

AGTTGCCTATTCAAACAGCTTCCACATTCTTAATTACTCTGTAAACCTCTCACAGAGTATTGATAAAGAAAC
► nValAlaTyrSerAsnSerPheHisIleLeuAsnTyrSerValAsnLeuSerGlnSerIleAspLysGluTh
GGGCAAACGTGACAACAGCATTTATTTAAGTCTCAGCCTGCCATTAGGCGATAACCATTCTGCAGATAGTAG
► rGlyLysArgAspAsnSerIleTyrLeuSerLeuSerLeuProLeuGlyAspAsnHisSerAlaAspSerSe
TTATTCTCGCAGTGGTAACGATATTAACCAACGACTTGGCGTAAATGGCTCTTTTGGTGAACGTCATCAATG
► rTyrSerArgSerGlyAsnAspIleAsnGlnArgLeuGlyValAsnGlySerPheGlyGluArgHisGlnTr
GAGTTATGGTATTAACGCTTCACGCAATAATCAAGGCTATCGCAGTTATGACGGTAATCTTTCGCATAACAA
► pSerTyrGlyIleAsnAlaSerArgAsnAsnGlnGlyTyrArgSerTyrAspGlyAsnLeuSerHisAsnAs
TAGCATTGGTAGTTACCGTGCTTCTTATTCACGTGATAGCCTCAAAAATCGCTCCATCTCACTGGGCGCAAG
► nSerIleGlySerTyrArgAlaSerTyrSerArgAspSerLeuLysAsnArgSerIleSerLeuGlyAlaSe
CGGTGCTGTCGTGGCGCACAAACACGGTATTACCTTAAGCCAACCTGTTGGCGAAAGTTTTGCCATTATTCA
► rGlyAlaValValAlaHisLysHisGlyIleThrLeuSerGlnProValGlyGluSerPheAlaIleIleHi
CGCCAAAGATGCCGCAGGAGCAAAAGTGGAATCAGGTGCCAATGTGAGCCTTGATTATTTCGGCAATGCGGT
► sAlaLysAspAlaAlaGlyAlaLysValGluSerGlyAlaAsnValSerLeuAspTyrPheGlyAsnAlaVa
TATGCCTTACACCAGCCCGTATGAAATCAATTATATCGGTATCAATCCATCTGATGCGGAGGCGAATGTGGA
► lMetProTyrThrSerProTyrGluIleAsnTyrIleGlyIleAsnProSerAspAlaGluAlaAsnValGl
ATTTGAAGCCACTGAACGCCAAATCATTCCTCGTGCAAATTCAATTAGCTTAGTAGATTTCCGCACGGGCAA
► uPheGluAlaThrGluArgGlnIleIleProArgAlaAsnSerIleSerLeuValAspPheArgThrGlyLy
AAATACAATGGTGTTATTTAACCTCACTTTGCCAAATGGCGAGCCAGTGCCAATGGCATCCACCGCACAAGA
► sAsnThrMetValLeuPheAsnLeuThrLeuProAsnGlyGluProValProMetAlaSerThrAlaGlnAs
TAGCGAAGGGGCATTTGTGGGCGATGTGGTGCAAGGTGGTGTGCTTTTCGCTAATAAACTTACCCAGCCAAA
► pSerGluGlyAlaPheValGlyAspValValGlnGlyGlyValLeuPheAlaAsnLysLeuThrGlnProLy
AGGCGAGTTAATCGTCAAATGGGGTGAGCGAGAAAGCGAACAATGCCGTTTCCAATATCAAGTTGATTTGGA
► sGlyGluLeuIleValLysTrpGlyGluArgGluSerGluGlnCysArgPheGlnTyrGlnValAspLeuAs
TAACGCACAAATACAAAGTCACGATATTCAATGCAAAACCGCAAAATAAATAATTGAAGAGGATTTATGCAA
► pAsnAlaGlnIleGlnSerHisAspIleGlnCysLysThrAlaLys ► MetGln
AAAACACCCAAAAAATTAACCGCGCTTTTCCATCAAAAATCCACTGCTACTTGTAGTGGAGCAAATTATAGT
► LysThrProLysLysLeuThrAlaLeuPheHisGlnLysSerThrAlaThrCysSerGlyAlaAsnTyrSer
GGAGCAAATTATAGTGGCTCAAAATGCTTTAGGTTTCATCGTCTGGCTCTGCTTGCTTGCGTGGCTCTGCTT
► GlyAlaAsnTyrSerGlySerLysCysPheArgPheHisArgLeuAlaLeuLeuAlaCysValAlaLeuLeu
GATTGCATTGTGGCACTGCCTGCTTATGCTTACGATGGCAGAGTGACCTTTCAAGGGGAGATTTTAAGTGAT
► AspCysIleValAlaLeuProAlaTyrAlaTyrAspGlyArgValThrPheGlnGlyGluIleLeuSerAsp
GGCACTTGTAAAATTGAAACAGACAGCCAAAATCGCACGGTTACCCTGCCAACAGTGGGAAAAGCTAATTTA
► GlyThrCysLysIleGluThrAspSerGlnAsnArgThrValThrLeuProThrValGlyLysAlaAsnLeu
AGCCACGCAGGGCAAACCGCCGCCCCTGTGCCTTTTTCCATCACGTTAAAAGAATGCAATGCAGATGATGCT
► SerHisAlaGlyGlnThrAlaAlaProValProPheSerIleThrLeuLysGluCysAsnAlaAspAspAla
ATGAAAGCTAATCTGCTATTTAAAGGGGGAGACAACACAACAGGGCAATCTTATCTTTCCAATAAGGCAGGC
► MetLysAlaAsnLeuLeuPheLysGlyGlyAspAsnThrThrGlyGlnSerTyrLeuSerAsnLysAlaGly

TABLE 5 (cont.)

AACGGCAAAGCCACCAACGTGGGCATTCAAATTGTCAAAGCCGATGGCATAGGCACGCCTATCAAGGTGGAC
► AsnGlyLysAlaThrAsnValGlyIleGlnIleValLysAlaAspGlyIleGlyThrProIleLysValAsp
GGCACCGAAGCCAACAGCGAAAAAGCCCCCGACACAGGTAAAGCGCAAAACGGCACAGTTATTCAACCCCGT
► GlyThrGluAlaAsnSerGluLysAlaProAspThrGlyLysAlaGlnAsnGlyThrValIleGlnProArg
TTTGGCTACTTTGGCTCGTTATTACGCCACAGGTGAAGCCACCGCAGGCGACGTTGAAGCCACTGCAACTTT
► PheGlyTyrPheGlySerLeuLeuArgHisArg
TGAAGTGCAGTATAACTAAAATATTTATTATCCAGTGAAAAAATGAATAAGAAATCGTATATAAATCATTAC
► MetAsnLysLysSerTyrIleAsnHisTyr

TTAACTTTATTTAAAGTTACTACTTTACTATTTACTCTTTCAAGTAATCCTGTATGGGCAAATATAAAAACA
► LeuThrLeuPheLysValThrThrLeuLeuPheThrLeuSerSerAsnProValTrpAlaAsnIleLysThr
GTTCAGGGAACAACTAGTGGTTTTCCACTTCTAACAAGAACTTTCACATTTAATGGCAATTTGCAATGGAAT
► ValGlnGlyThrThrSerGlyPheProLeuLeuThrArgThrPheThrPheAsnGlyAsnLeuGlnTrpAsn
GTGAGTGCTCTACAACCAGCTTATATTGTTTCCTCTCAAGCAAGAGATAATCTTGATACAGTACATATTCAA
► ValSerAlaLeuGlnProAlaTyrIleValSerSerGlnAlaArgAspAsnLeuAspThrValHisIleGln
TCTTCTGAAATTAATGCTCCAACAAATTCATTAGCTCCATTTAATAATTGGATTAATACGAAATCAGCAGTA
► SerSerGluIleAsnAlaProThrAsnSerLeuAlaProPheAsnAsnTrpIleAsnThrLysSerAlaVal
GAGCTAGGTTATAGCTTTGCGGGCATTACTTGTACTAGTAATCCTTGCCCAACAATGAAATTACCATTATTA
► GluLeuGlyTyrSerPheAlaGlyIleThrCysThrSerAsnProCysProThrMetLysLeuProLeuLeu
TTTCATCCTGATCTTACTAATTTAACTCCACCTGGAAAGAAAAATTCTGATGGAGGGGAGATTTTTAAATTA
► PheHisProAspLeuThrAsnLeuThrProProGlyLysLysAsnSerAspGlyGlyGluIlePheLysLeu
CATAATGAATCTAATTTAGGCGTCTCTTTTCAAATTGGAGTAAAAACGAATACTTCTCTAGATTGGGTTAAT
► HisAsnGluSerAsnLeuGlyValSerPheGlnIleGlyValLysThrAsnThrSerLeuAspTrpValAsn
GCTAAGAATAATTTTAGCTCTCTAAAAGTTTTAATGGTGCCTTTTAATTCTAGCGATAAAATATCTTTGCAT
► AlaLysAsnAsnPheSerSerLeuLysValLeuMetValProPheAsnSerSerAspLysIleSerLeuHis
TTACGTGCTAAATTTCATTTATTAACAGATTTTTCATCGCTAAATAATGATATTACTATTGACCCTATGAAT
► LeuArgAlaLysPheHisLeuLeuThrAspPheSerSerLeuAsnAsnAspIleThrIleAspProMetAsn
ACTAGTATAGGCAAAATTAATCTTGAAACGTGGCGTGGCTCAACAGGCAATTTTTCTGTTAAATATGTAGGT
► ThrSerIleGlyLysIleAsnLeuGluThrTrpArgGlySerThrGlyAsnPheSerValLysTyrValGly
GAGGATAAGGGAGATATATCTATTTTCTTTAATACACCTAAAATTATTCTAAAAAAACAACAACGCCGATGT
► GluAspLysGlyAspIleSerIlePhePheAsnThrProLysIleIleLeuLysLysGlnGlnArgArgCys
ACTCTGAATAATGCTCCAGTGAGCCCAAATCCAGTTAAATTACGAGCGGTAAAAAAACGTGAATTGGAGGCA
► ThrLeuAsnAsnAlaProValSerProAsnProValLysLeuArgAlaValLysLysArgGluLeuGluAla
CAAAGTGAAATGGAAGGTGGGACATTTCAGTTAAGAGTAAATTGTGACAATACCACTTATAATAAAGCCAAC
► GlnSerGluMetGluGlyGlyThrPheGlnLeuArgValAsnCysAspAsnThrThrTyrAsnLysAlaAsn
GGCAAATGGTTATTTCCTGTAGTGAAAGTTACTTTTACGGACGAAGATGGTACAACGAATAATGGAACAAAT
► GlyLysTrpLeuPheProValValLysValThrPheThrAspGluAspGlyThrThrAsnAsnGlyThrAsn

TABLE 5 (cont.)

GACTTACTTCGCACCCAAACAGGCAGCGGACAAGCCACAGGCGTTAGCTTAAGAATCAAACGAGAAAATGGT
► AspLeuLeuArgThrGlnThrGlySerGlyGlnAlaThrGlyValSerLeuArgIleLysArgGluAsnGly
ACAGAAACCGTAAAATACGGTGCTGATTCTGCTCAAATGGGGAATGCTGGACAATTTGAATTACGAAAACAA
► ThrGluThrValLysTyrGlyAlaAspSerAlaGlnMetGlyAsnAlaGlyGlnPheGluLeuArgLysGln
CCATCCCCTGCTGGTGGAGATCAATATGCTGAAGAAACTTTCAAAGTCTATTACGTAAAAGACTCAACAAGA
► ProSerProAlaGlyGlyAspGlnTyrAlaGluGluThrPheLysValTyrTyrValLysAspSerThrArg
GGCACCTTAATCGAAGGAAAAGTCAAAGCCGCCGCCACTTTCACAATGTCATATCAATAATAATGTCGGGTG
► GlyThrLeuIleGluGlyLysValLysAlaAlaAlaThrPheThrMetSerTyrGln
GGAATATAAAGGCTGAAGGTTTAAACTTCAGTCTTTTTTATAGGAAAATACCATTGCAACTTTAAGGATAA
AATTTTATCCTAAGCACAATTTTTATAAGAATAGGTCAAATTATGTTAGCCAAAGCAAAATATAGAAAAGAT
► MetLeuAlaLysAlaLysTyrArgLysAsp
TACAAACAACCAGATTTTACGGTCACAGACATTTATTTAGATTTTCAACTTGATCCTAAAAATACTGTGGTG
► TyrLysGlnProAspPheThrValThrAspIleTyrLeuAspPheGlnLeuAspProLysAsnThrValVal

ACTGCAACCACAAAATTCCAACGCTTAAATAATGAAGCGACGTCTTTACGTTTAGACGGGCATAGCTTCCAG
► ThrAlaThrThrLysPheGlnArgLeuAsnAsnGluAlaThrSerLeuArgLeuAspGlyHisSerPheGln
TTTTCTTCTATTAAATTTAATGGCGAGCCATTTTCTGATTATCAACAAGATGGCGAGAGTTTAACGCTCGAT
► PheSerSerIleLysPheAsnGlyGluProPheSerAspTyrGlnGlnAspGlyGluSerLeuThrLeuAsp
TTAAAAGACAAAAGTGCGGATGAATTTGAGCTTGAAATTGTGACGTTCCTTGTGCCAGCCGAAAATACGTCA
► LeuLysAspLysSerAlaAspGluPheGluLeuGluIleValThrPheLeuValProAlaGluAsnThrSer
TTACAAGGGCTATATCAGTCTGGCGAAGGTATTTGTACGCAATGTGAGGCGGAAGGTTTCCGTCAAATCACT
► LeuGlnGlyLeuTyrGlnSerGlyGluGlyIleCysThrGlnCysGluAlaGluGlyPheArgGlnIleThr
TATATGCTTGATCGTCCTGATGTGCTGGCGCGTTATATAATCAAAATTACGGCAGATAAAACCAAATATCCA
► TyrMetLeuAspArgProAspValLeuAlaArgTyrIleIleLysIleThrAlaAspLysThrLysTyrPro
TTCTTACTGTCGAATGGTAATCGCATTGCAAGTGGCGAATTAGAAGATGGTCGCCATTGGGTGGAATGGAAT
► PheLeuLeuSerAsnGlyAsnArgIleAlaSerGlyGluLeuGluAspGlyArgHisTrpValGluTrpAsn
GATCCTTTCCCAAAACCAAGCTATTTATTTGCTTTAGTGGCGGGAGATTNNGGTTTATTACAAGATAANTTT
► AspProPheProLysProSerTyrLeuPheAlaLeuValAlaGlyAspXaaGlyLeuLeuGlnAspXaaPhe
ATTACTAAAAGTGGTCGTGAAGTGGCTTTAGAGCTTTATGTGGATCGCGGTAATCTTAACCGTGCAACTGGG
► IleThrLysSerGlyArgGluValAlaLeuGluLeuTyrValAspArgGlyAsnLeuAsnArgAlaThrGly
GCAATGGAAAGTCTGAAAAAAGCGATGAAATGGGATGAAGATCGCTTTATTTTAGAATTTTACCTAGATATT
► AlaMetGluSerLeuLysLysAlaMetLysTrpAspGluAspArgPheIleLeuGluPheTyrLeuAspIle
TATATGATCGCGGCCGCCGATTCCTCCAATATGGGCGCAATGGAAAATAAAGGATTAAATATCTTTAACTCT
► TyrMetIleAlaAlaAlaAspSerSerAsnMetGlyAlaMetGluAsnLysGlyLeuAsnIlePheAsnSer
AAATTGGTGTTGGCAAATCCACAAACGGCAACAGATGAAGATTATCTTGTCATTGAAAGTGTGATTGCACAC
► LysLeuValLeuAlaAsnProGlnThrAlaThrAspGluAspTyrLeuValIleGluSerValIleAlaHis

TABLE 5 (cont.)

GAATATTCCCATAACTGGACGGGAAACCGTGTAACCCGCCGAGATGGGTTCAACTAGGTTTGAAGAAGGTTA

► GluTyrSerHisAsnTrpThrGlyAsnArgValThrArgArgAspGlyPheAsn

ACGGCTTCCGGGAACAAGATTTCTCAGATCAGTTCTCCGGGCCGGAACCGATTAATAAGGGAAAATTTTCCG

CLAIMS

1. An isolated DNA sequence selected from the group consisting of:
   (i) an isolated DNA sequence comprising the nontypable *Haemophilus influenzae* serotype 1 LKP operon selected from the group consisting of:
      a) SEQ ID NO: 4;
      b) the complementary strand of SEQ ID NO: 4; and
      c) DNA sequences that hybridize to SEQ ID NO: 4;
   (ii) an isolated DNA sequence comprising the *Haemophilus influenzae* serotype 1 LKP *hipP* gene selected from the group consisting of:
      a) nucleotides 1882 to 2532 of SEQ ID NO: 4;
      b) the complementary strand of a); and
      c) DNA sequences that hybridize to a);
   (iii) an isolated DNA sequence comprising the *Haemophilus influenzae* serotype 1 LKP *hipC* gene selected from the group consisting of:
      a) nucleotides 2854 to 3636 of SEQ ID NO: 4;
      b) the complementary strand of a); and
      c) DNA sequences that hybridize to a);
   (iv) an isolated DNA sequence comprising the *Haemophilus influenzae* serotype 1 LKP *hipR* gene selected from the group consisting of:
      a) nucleotides 4016 to 6238 of SEQ ID NO: 4;
      b) the complementary strand of a); and
      c) DNA sequences that hybridize to a);
   (v) an isolated DNA sequence comprising the *Haemophilus influenzae* serotype 1 LKP *hipM* gene selected from the group consisting of:
      a) nucleotides 6259 to 6873 of SEQ ID NO: 4;
      b) the complementary strand of a); and

c) DNA sequences that hybridize to a);

(vi) an isolated DNA sequence comprising the *Haemophilus influenzae* serotype 1 LKP *hipA* gene selected from the group consisting of:

a) nucleotides 6955 to 8365 of SEQ ID NO: 4;

b) the complementary strand of a); and

c) DNA sequences that hybridize to a); and

(vii) an isolated DNA sequence comprising the *Haemophilus influenzae* serotype 1 LKP tip adhesin comprising nucleotides 6955 to 8265 of SEQ ID NO: 4.

2. An isolated *Haemophilus influenzae* protein, selected from the group consisting of:

(a) a serotype 1 LKP pilin protein, having the amino acid sequence comprising SEQ ID NO: 5;

(b) a serotype 1 LKP periplasmic chaperone protein having the amino acid sequence comprising SEQ ID NO: 6;

(c) a serotype 1 LKP membrane anchor protein having the amino acid sequence comprising SEQ ID NO: 7;

(d) a serotype 1 LKP tip associated protein having the amino acid sequence comprising SEQ ID NO: 8;

(e) a serotype 1 LKP tip adhesin protein having the amino acid sequence comprising SEQ ID NO: 9; or

(f) a serotype 5 LKP pilin protein having the amino acid sequence comprising SEQ ID NO: 3; or

biologically active fragments thereof.

3. A recombinant *Haemophilus influenzae* LKP tip adhesin fusion protein comprising a biologically fragment of the LKP tip adhesin protein and maltose binding protein or glutathione-S-transferase.

4. A recombinant *Haemophilus influenzae* serotype 1 LKP tip adhesin fusion protein having the amino acid sequence comprising SEQ ID NO: 11, or a biologically active fragment thereof.

5. A method of producing an antibody which binds to *Haemophilus influenzae* LKP tip adhesin comprising immunizing a mammal with a recombinant *Haemophilus influenzae* LKP tip adhesin fusion protein.

6. A recombinant expression vector comprising a DNA insert encoding a *Haemophilus influenzae* LKP pilus, said expression vector capable of expressing a biologically active LKP pilus in a procaryotic or eucaryotic cell.

7. A procaryotic or eucaryotic host cell transformed with an expression vector comprising a DNA insert encoding a *Haemophilus influenzae* LKP pilus, said expression vector capable of expressing a biologically active LKP pilus in the host cell.

8. A method of producing *Haemophilus influenzae* serotype 10, serotype 11 or serotype 12 LKP pilus in a procaryotic or eucaryotic host cell comprising the steps of:

a) inserting a DNA sequence encoding a serotype 10, serotype 11 or serotype 12 LKP pilus into an expression vector capable of expressing a biologically active LKP pilus in the host cell, thereby producing a LKP pilus expression vector; and

b) transfecting an appropriate host cell with the expression vector produced in step a) and maintaining the transfected host cell under

conditions suitable for the expression of a LKP pilus in the host cell.

9. A DNA probe capable of hybridizing to a DNA sequence encoding the *Haemophilus influenzae* tip adhesin protein comprising nucleotides between about 6955 to 8265 of SEQ ID NO: 4.

10. A recombinant DNA molecule which encodes a *Haemophilus influenzae* tip adhesin protein or active fragment thereof.

11. A method of assaying for the presence of *Haemophilus influenzae* in a biological sample comprising the steps of contacting the sample with a DNA probe comprising about 400 nucleotides capable of hybridizing to a DNA sequence encoding a tip adhesin protein under conditions suitable for hybridization and detecting the presence of hybridized DNA.

12. A method of vaccinating a mammal against *Haemophilus influenzae* comprising administering to the mammal an effective amount of *Haemophilus influenzae* LKP tip adhesin protein, or a biologically active fragment thereof.

13. A method of protecting mammals against infections caused by *Haemophilus influenzae* comprising administering to the mammal an effective amount of a composition comprising *Haemophilus influenzae* LKP tip adhesin protein, or a biologically active fragment thereof, and a pharmaceutically acceptable carrier.

14. A vaccine composition for protecting mammals against infections caused by *Haemophilus influenzae* comprising a pharmaceutically acceptable carrier and *Haemophilus influenzae* LKP tip adhesin protein, or a biologically active fragment thereof.

```
                    10         20         30         40         50         60         70
                *    *    *    *    *    *    *    *    *    *    *    *    *    *
LKP1 hifA    MKKTTTGSLI LLAFATNAAD PQVSTETSGK VTFFGKVVEN TCKVKTDSKN MSVVLNDVGK NHLKTKKDTA
LKP4 hifA           10         20        |30        |40        |50        |60
             ....LL.... .....G.VQA -DIN...... .......... ......EH.. L......... .S.S..VN..>
                                    N
LKP5 hifA           10         20    |     30|        40|        50|        60|        70|
             ....LL.... .....G.VQA ADPNP..K.. ...Y...... .....SGNRD .......... A..SQ.GY..>

                    80         90        100        110        120        130        140
                *    *    *    *    *    *    *    *    *    *    *    *    *    *
LKP1 hifA    MPTPFTINLE NCSTTTTTNN KPVATKVGAY FYSWKNADEN NEYTLKNTKS GNDAAQNVNI QTFDANGTDA
LKP4 hifA 70          |80        |90        100        110        120        130
             .......T.Q ..DP..ANGT ANK.N...L. ......V.KE .NF....EQT TA.Y.T.... .LMES...K.>
LKP5 hifA        80|        |90        100        110        120        130
             .......T.. G.NAN.G.-- ..K.N...V. ....N...KE .S....S.LT .T.K.D.... .I.QE.....>

                   150        160        170        180        190        200        210
                *    *    *    *    *    *    *    *    *    *    *    *    *    *
LKP1 hifA    IEVVGNGTTD FTHSNTNDVA TQQTVNKNHI SGKATINGEN NVKLHYIARY YATAQAEAGK VESSVDFQIA
                                     H               T
LKP4 hifA 140       150        160  |     170       |180|       190|       200|       210|
             .S...KE.E. .M.T.N.G.. LN..P.NT.. ..STQLT.T. ELP..F..Q. ...NK.T... .Q........>
                                                       T
LKP5 hifA 140       150        160       |170       |180        190        200
            .G.ADKTID. ...K.NGSTN SDKP-T.... .SATAL.NQG DIA.....Q. ...GM.S... GPT....P..>

LKP1 hifA    YE*
LKP4 hifA
               ..*
LKP5 hifA   210
              .*
```

Figure 1

CLJ11

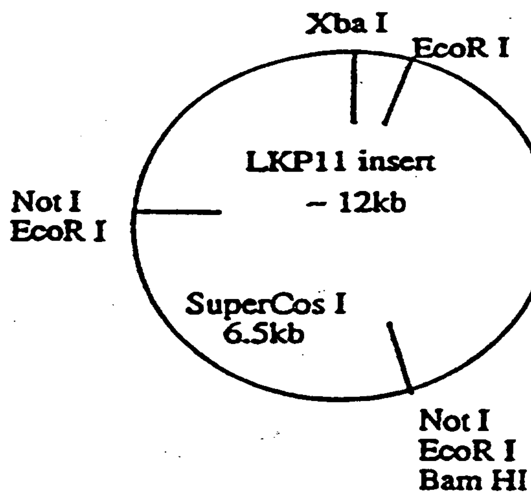

Fig. 2A

CLJ10

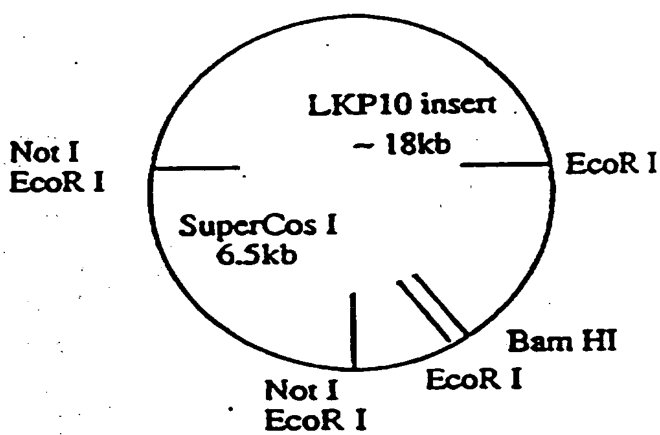

Fig. 2B

CLJ12

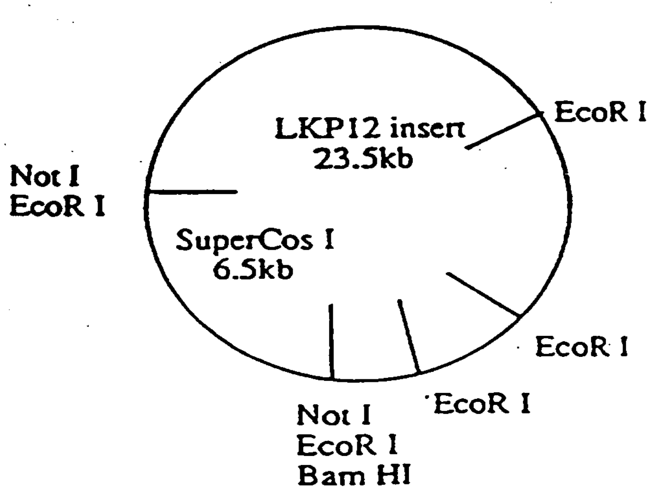

Fig. 2C

KIEEGKLVIWINGDKGYNGLAEVGKKFEKDTGIKVTVEHPDKLEEKFPQVAATGDGPDIIF
WAHDRFGGYAQSGLLAEITPDKAFQDKLYPFTWDAVRYNGKLIAYPIAVEALSLIYNKDLL
PNPPKTWEEIPALDKELKAKGKSALMFNLQEPYFTWPLIAADGGYAFKYENGKYDKIKDVG
VDNAGAKAGLTFLVDLIKNKHMNADTDYSIAEAAFNKGETAMTINGPWAWSNIDTSKVN
YGVTVLPTFKGQPSKPFVGVLSAGINAASPNKELAKEFLENYLLTDEGLEAVNKDKPLGA
VALKSYEEELAKDPRIAATMENAQKGEIMPNIPQMSAFWYAVRTAVINAASGRQTVDEALK
DAQTRITKIEGR<u>TLSSNPVWANIKTVQGTTSGFPLLTRTFTENGNLQWNVSALQPAYIVSSQ</u>
<u>ARDNLDTVHIQSSEINAPTNSLAPENNWINTKSAVELGYSFAGITCTSNPCPTMKLPLLFHP</u>
<u>QLTNLTPPGKKNSDGGEIFKLHNESNLGVSFQIGVKTNTSLDWVNAKNNFSSLKVLMVPF</u>
<u>NSSKSISLHLRAKFHLLTDFSSLNNDITIDPMNTSIGKINLETWRGSTGNFSVKYVGEDKG</u>
<u>DISIFFNTPKIILKKQQRRCTLNNAPVSPNPVKLRAVKKRELEAQSEMEGGTFQLRVNCDN</u>
<u>TTYNKAN</u>

Fig. 3

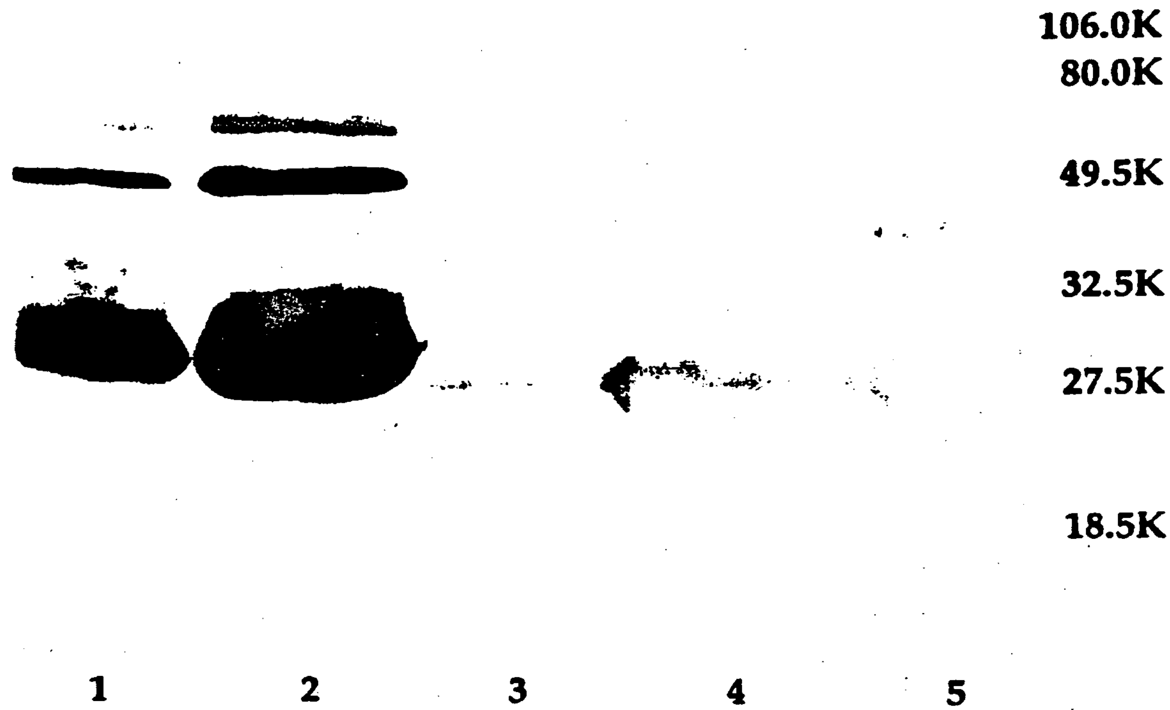

FIG. 4

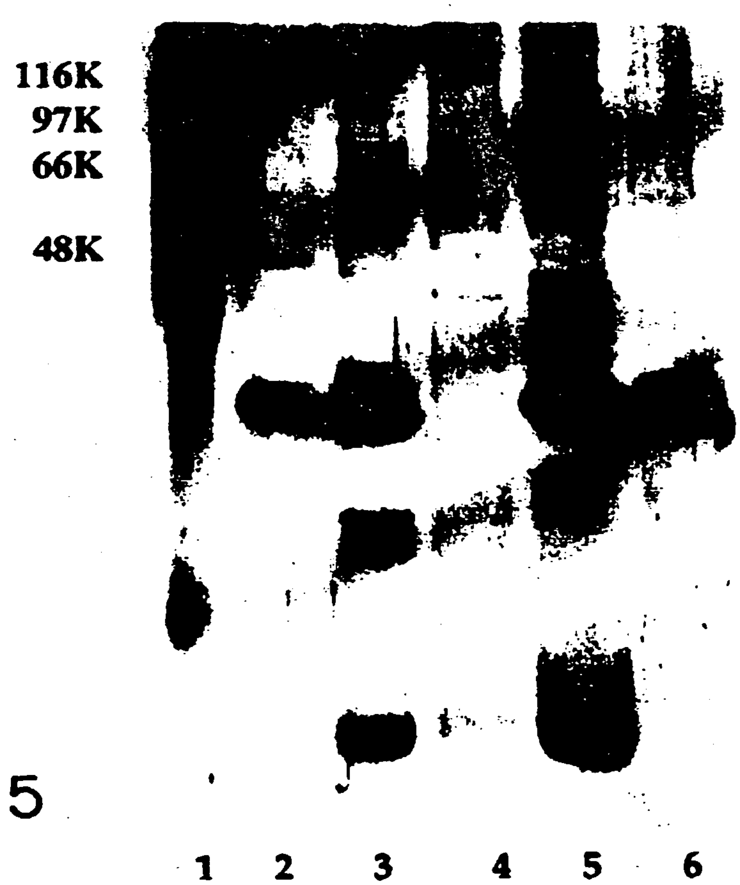

FIG. 5

FIG. 6

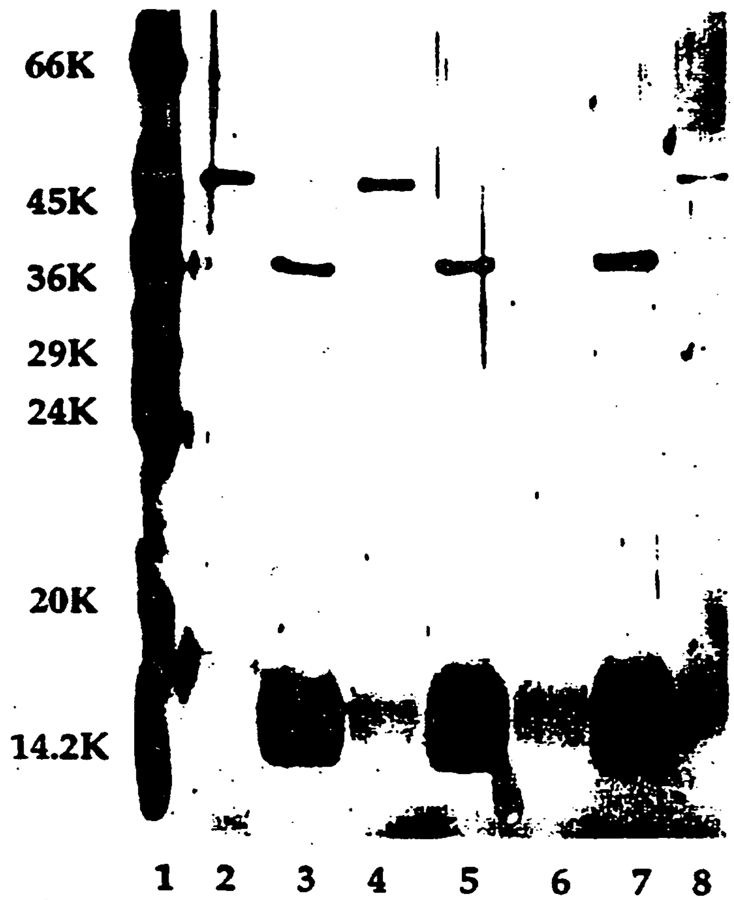
66K
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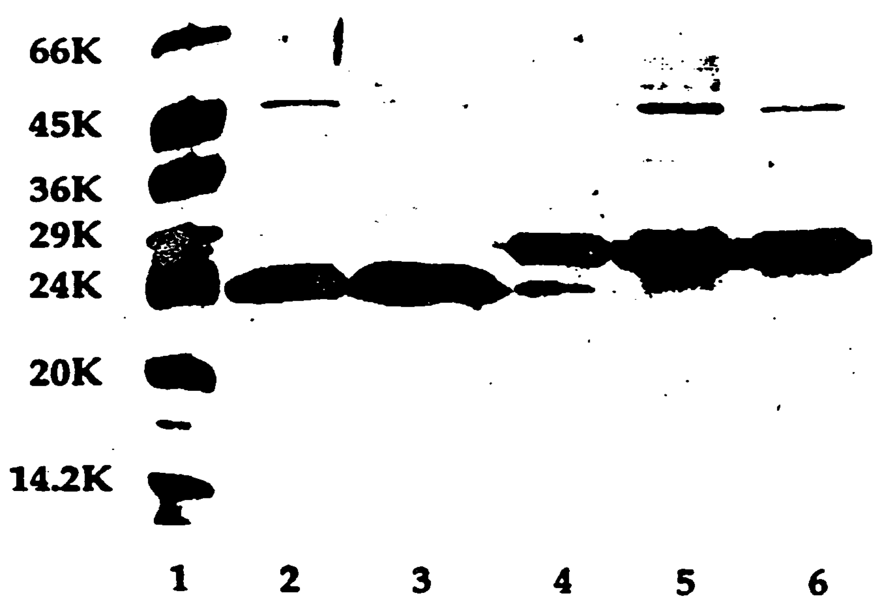
FIG. 7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1 2 3 4 5 6

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 95/08789

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C12N 15/31, C07K 14/285, A61K 39/102, C12N 1/21, C12N 5/10, C12Q 1/68
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C12N, A61K, C07K, C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI, EDOC, MEDLINE, BIOSIS, DERWENT BIOTECHNOLOGY ABSTRACT

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO, A1, 9319090 (BARENKAMP, STEPHEN, J.), 30 Sept 1993 (30.09.93), page 5, line 9 - line 36 --	1-11
X	US, A, 5336490 (CHARLES C. BRINTON, JR. ET AL), 9 August 1994 (09.08.94), column 2, line 30 - line 63, claims	1-8
A	 --	9-11
X	Database EMBL/GenBank/DDBJ on STRAND,ID=HIIFC, AC=U02932, Watson et al: "Identification of a Gene essential for Piliation in Haemophilus influenzae type b with Homology to the Pilus Assembly Platform Genes of Gram-Negative Bacteria", 1994. --	1-2

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" ertier document but published on or after the international filing date
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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
30 November 1995	11.01.96

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL-2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Patrick Andersson

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 95/08789

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Database EMBL/GenBank/DDBJ on STRAND,ID=HIHIFB, AC=X66606, Smith AL et al: "hif B of H. influenzae is a member of the chaperone family", 1992. --	1-2
X	Database EMBL/GenBank/DDBJ on STRAND,ID=HIHIIFA, AC=X16991, van Ham SM et al: "Cloning and expression in Escherichia coli of Haemophilus influenzae fimbral genes establisches adherence to oropharyngeal epithelial cells", EMBO J., vol 8,p3535, 1989. --	1-12
P,X	Database EMBL/GenBank/DDBJ on STRAND, ID=HII9795, AC=U19795, Green BA et al: "Sequence of LKP serotype 5 hifA gene", 1995. --	1-2

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 95/08789

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 12-14
because they relate to subject matter not required to be searched by this Authority, namely:
See PCT Rule 39.1(iv): Methods for treatment of the human or animal body by surgery or therapy, as well as diagnostic methods.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest
[ ] The additional search fees were accompanied by the applicant's protest.
[ ] No protest accompanied the payment of additional search fees.

"A 114511

INTERNATIONAL SEARCH REPORT

Information on patent family members

30/10/95

International application No.

PCT/US 95/08789

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A1- 9319090	30/09/93	AU-A- 3916893	21/10/93
		EP-A- 0632814	11/01/95
		FI-A,D- 944273	15/11/94
		JP-T- 7506248	13/07/95
		NO-A,D- 943431	10/11/94
US-A- 5336490	09/08/94	AU-A- 3772389	12/01/90
		WO-A- 8912460	28/12/89

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